European *Solanum dulcamara* L. and its interaction with *Phytophthora infestans* (Mont.) de Bary

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General introduction

***Solanum dulcamara* L.**

Solanum dulcamara, commonly named bittersweet, owes its name from Latin *dulcis* (= sweet) and *amarus* (= bitter) what refers to the taste of the plant, that is said to be, first bitter and then sweet. Bittersweet is a perennial, straggling or climbing vine, somewhat woody below and with branches up to 3 meters. The leaves are ovate to hastate and lobed near the base. Flowers, with exerted style and yellow anthers are blue, rarely white and grouped in lateral compound cymes containing 20-25 flowers. The berry is bright red, oval to globose with about twenty light colored minutely reticulate seeds (Figure 1).

Figure 1. Drawing of *S. dulcamara* (reprinted from Flora von Deutschland Österreich und der Schweiz (1885)).

This diploid Old World species is also widely naturalized in the temperate New World areas. *Solanum dulcamara* flowers in summer and can be found in different habitats: damp woods, lake borders, hedges, river banks, dunes, lake shores and waste places.

Taxonomic relationship of *S. dulcamara*

Solanum dulcamara is one of the about 1500 species of the genus *Solanum*. Weese and Bohs (2007) in their phylogeny study placed *S. dulcamara* in the Dulcamaroid clade which is closely related to the Morelloid clade with *Solanum* section *Solanum* species as *S. ptychanthum* Dunal and *S. villosum* Mill. (Figure 2).

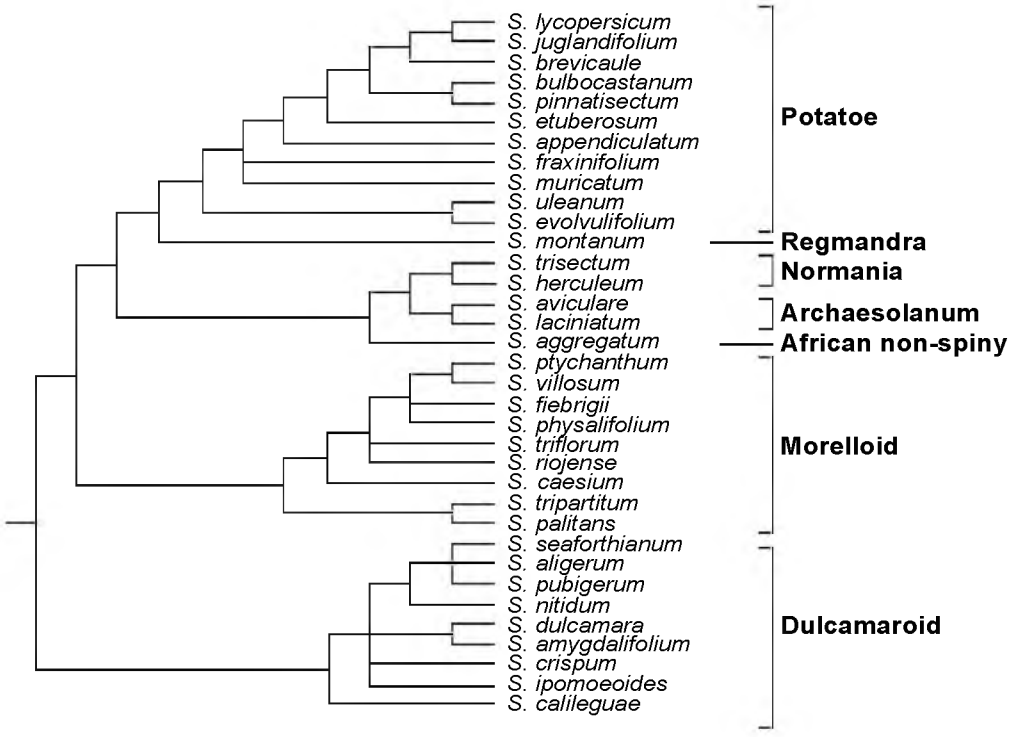

Figure 2. Part of the tree representing the phylogeny of *Solanum* species related to *S. dulcamara* (reprinted from Weese and Bohs 2007).

Figure 2 also shows the clear relationship between the Dulcamaroid clade and the Potatoe clade. According to the more traditional classification *S. dulcamara* is designated as the type for the section *Dulcamara* (Moench) Dumort. The section *Dulcamara* as well as the sections *Petota* Dumort. and *Lycopersicum* (Tourn.) Wettst. belong to the subgenus *Potatoe* (G.Don) D'Arcy (Lester 1991; Child and Lester 2001). This classification shows the relationship between *S. dulcamara* and the potato, *S. tuberosum* L., which is in the section *Petota*.

Solanum dulcamara – alternative host

Solanum dulcamara is one of the few native species of *Solanum* present in Europe recently however, it has been widely naturalized around the world (Figure 3).

Figure 3. Map of the world presenting geographical occurrence of *S. dulcamara*.

Together with other *Solanum* species present in Europe, like *S. nigrum* L., it is considered as a weed of arable fields and the alternative host for diseases that attack cultivated potato. In previous decades scientists discovered that bittersweet is a host for the quarantine pathogen *Ralstonia solanacearum* (Smith) Smith, a causal agent of bacterial wilt or brown rot of potato. It was proven that *R. solanacearum* is able to survive and to develop on the roots of *S. dulcamara* that grows in ditches, from which, water was used to irrigate adjacent potato fields (Olsson 1976; Elphinstone et al. 1996; Janse 1996). Since then it is forbidden to use surface water for this purpose and in some places attempts were made to eradicate *S. dulcamara* from potato cultivation areas (Persson 1998).

Solanum dulcamara is also a host for *Phytophthora infestans* (Mont.) de Bary (de Bary 1876; Cooke et al. 2002; Flier et al. 2003; Dandurand et al. 2006), but infections are rarely observed. In nature, this plant species might help *P. infestans* to survive between cropping seasons while potato is not present. On the other hand, possibly bittersweet offers a new pool of resistance genes, yet unknown and not characterized, that can in future help to protect potato. In this respect bittersweet is still poorly understood and seldom investigated. Its evident easiness of genetic system, reproduction, maintenance and abundance in nature should possibly draw more attention of scientific community in the future.

Phytophthora infestans – pathogen of the potato

The oomycete *P. infestans* is the causal agent of late blight, the most important disease of cultivated potato known today (Fry 2007). It was first reported in 1844 when spot infections were observed near Liège (Belgium), in Kent, the Isle of Man and probably in Ireland.

Due to the *P. infestans* favorable weather conditions in 1845 it spread rapidly from Belgium over Europe destroying the potato fields from Spain to the Scandinavian countries and from Italy to Ireland (Butler and Jones 1955) and causing the "Irish famine" (Large 1940).

In 1846 again the weather was favoring *Phytophthora* growth and the potato harvest failed because plants and tubers were destroyed. This event caused a subsequent famine that many people died or survivors immigrated to North America (Agrios 2005). Since then it is a major threat and main concern in potato production. It took 16 years after the "Irish famine" till Anton de Bary proved that potato blight was caused by a fungus (Agrios 2005). *Phytophthora infestans* is a heterothallic species with two mating types: A1 and A2 (Niederhauser 1956; Gallegly and Galindo 1958). However until 1980's only the A1 mating type, having low genetic variation, was present worldwide. Subsequently, A2 migrated, enabling sexual reproduction and increasing genetic variability of the pathogen. Since then the host range of this pathogen has changed drastically as a result of greater aggressiveness both on cultivated potato, tomato and wild *Solanum* species (Fry et al. 1993, Goodwin et al. 1994).

Potato – host of *Phytophthora infestans*

The history of potato began in the Andean region of Peru and Bolivia where it was cultivated by natives for thousands of years. However, it never became a major food source (Hawkes 1990). When it was brought to Europe in 16th century by Spanish explores it was quickly distributed to other countries of Europe and subsequently to the rest of the world, where in short time it became most important crop for millions of people providing nutritious food, growing faster and with higher yield than any other crop. Potato tuber is now the fourth most important crop – after maize, wheat and rice - with annual production around 320 millions tons in year 2007 (FAOSTAT 2007). Production of tubers is growing steadily for the last two decades mainly because of an increase in the area used for cultivation especially in Asia (India, China) and thanks to progress made in agriculture. In the last four decades the average yield of potato tubers in developed countries of Western Europe rose from 18 tons/ha in 1960 to 40 tons/ha in 2000. Still there exists a huge demand from industry for processed potato products (French fries, chips, pre-cooked products and starch products) that will still require increase of production in the coming years.

Diseases of potato

Besides late blight, potato is threatened by numerous pests and diseases attacking leaves, stems and roots during its growth and tubers during growth and storage (Table 1).

Table 1. Most important diseases and pests limiting potato tuber yield.

BACTERIAL DISEASES	
Bacterial wilt syn. brown rot	*Ralstonia solanacearum*
Blackleg and bacterial soft rot	*Erwinia carotovora* subsp. *atroseptica*
Ring rot	*Clavibacter michiganensis* subsp. *sepedonicus*
Common scab	*Streptomyces scabiei*
FUNGAL DISEASES	
Late blight	*Phytophthora infestans*
Early blight	*Alternaria solani*
Early dying disease	*Verticillium* spp.
Silver scab	*Helminthosporium solani*
Powdery scab	*Spongospora subterranea*
Black dot	*Colletotrichum coccodes*
Wart disease	*Synchytrium endobioticum*
Fusarium dry rot	*Fusarium* spp.
Brown spot and black pit	*Alternaria alternata*
PARASITIC NEMATODES	
Potato cyst	*Globodera pallida, Globodera rostochiensis*
Root knot nematode	*Meloidogyne* spp.
VIRAL DISEASES	
PVY	genus *Potyvirus,* Potato virus Y
PVX	genus *Potexvirus,* Potato virus X
PLRV	genus *Luteovirus,* Potato leafroll virus
PVS	genus *Potyvirus,* Potato virus S
PVA	genus *Potyvirus,* Potato virus A
PARASITIC INSECTS	
Colorado potato beetle	*Leptinotarsa decemlineata*
Potato tuber moth	*Phthorimaea operculella*

The potential production of potato tubers could reach the level of 400 millions tons worldwide if these diseases could be efficiently controlled (Agrios 1997). So far, chemical control, input of fertilizers, irrigation and the use of certified seed are the main way to obtain high yield.

Taxonomy of *Phytophthora infestans*

Oomycetes are a diverse group of eukaryotic fungal-like microorganisms. The genus *Phytophthora* consists of over 60 different species with most of them being plant pathogens. Oomycetes had been initially placed in the fungal kingdom because symptoms of late blight resemble those of fungal diseases, but were excluded on bases of their characteristics such as cell walls composed of cellulose and glycan rather than chitin, aseptate hyphae, food storage as starch and the presence of biflagellate swimming zoospores.

According to taxonomic studies *P. infestans* belongs to the order Peronosporales or downy mildews, Together with the genus *Pythium*, which is causing e.g. damping-off of seedlings and root rots, *Phytophthora* is a member of the Pythiaceae (Agrios 2005)

Origin

Speculations about the origin of the pathogen and the source of the initial inoculum began soon after the 1840s Irish famine. The 19th century naturalists concluded that the disease arrived from South America, because regions of Bolivia, Ecuador and Peru are the center of origin for the cultivated potato and solanaceous species. However, different theories arose that supported a Mexican place of birth for the pathogen rather than a South American one. This was concluded based on the presence of sexual reproduction combined with high nuclear diversity present among Mexican populations of *P. infestans* (Niederhauser 1991; Grünwald and Flier 2005).

Goodwin et al. (1994a) stated that the initial migration of *P. infestans* in the 1840s was from Mexico to the United States and that a single genetic individual was transported to Europe and subsequently to the rest of the world. More theories were raised like a three-step migration process, which states that the pathogen migrated from Mexico to South America, and subsequently from there to North America and Europe. Some historical evidence also suggests an ancient introduction (at least several centuries ago) of late blight into the Andean regions of South America from its original home in central Mexico (Andrivon 1996).

Abad and Abad (1997) showed that strong evidence exists in the historical literature for the disease being endemic in Peru and other Andean countries for centuries and before to the 1840's late blight epidemics started in Europe. This theory is supported also by little or no evidence for potato cultivation in Mexico prior to the Spanish conquest, as it was not widely cultivated there until 1949. The very first record of late blight on potatoes in Mexico was in 1908, thus, the introduction of the oomycete into Europe and the United States in the 1840s in cultivated potatoes from Mexico is highly improbable (Abad and Abad 1997).

Recently, evidence is accumulating that indeed South America is the center of origin for the pathogen, whereas Mexico should be considered as a center of diversity. Gomez-Alpizar et al. (2007) discovered at least two divergent ancestral lineages of the pathogen in the South American Andes and only one in Toluca Valley (Mexico). This is probably a result of a single genetic origin or founder effect from the South American source. Moreover extant haplotypes found in the Andes were derived from ancient lineages, whereas haplotypes found in the Toluca Valley were always derived from only one of the ancient lineages.

Although evidence suggests that Mexico is the source of recent migrations of *P. infestans* into Europe and to other areas of the world, earlier migrations of the pathogen more likely came from Peru.

Late blight – life cycle

Late blight caused by *P. infestans* in favorable conditions (high humidity and mild temperatures) is able to infect all parts of the host plant. The disease cycle (Figure 4) starts when sporangia release motile zoospores, which infect the host.

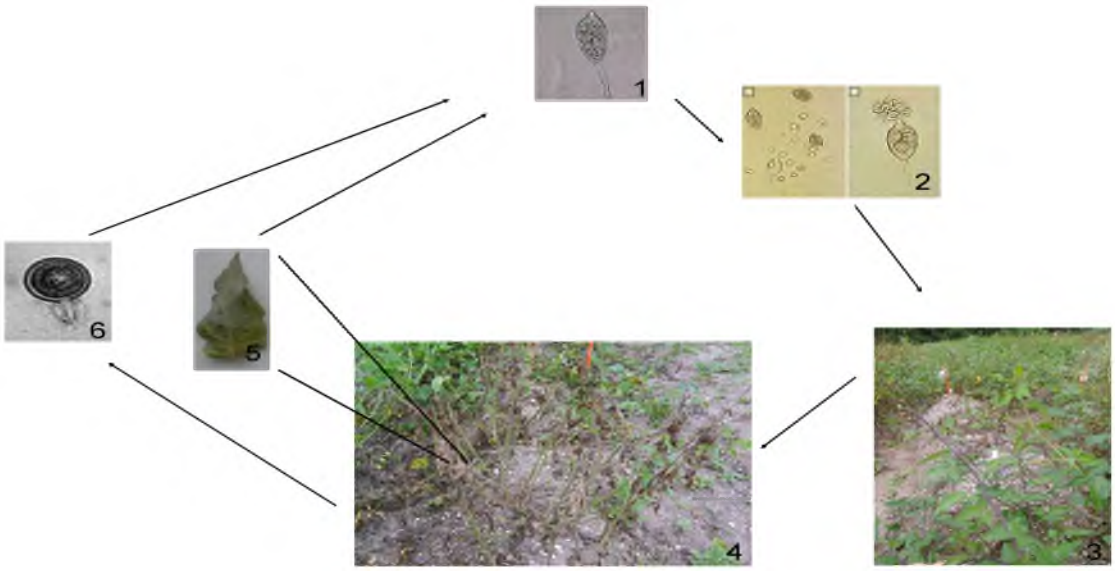

Figure 4. Life cycle of *Phytophthora infestans*, causal agent of potato late blight. Enlargement of sporangium (1) from which zoospores (2) are released upon favorable weather conditions and presence of the host (*Solanum dulcamara*) (3). Rapid colonization of the host tissues (4) by *P. infestans* with intensive sporulation (5) ends the asexual life cycle of the pathogen. In the presence of two opposing mating types, sexual reproduction occurs resulting in formation of oospores (6), bodies capable of winter survival.

From the infection site, the pathogen spreads, rapidly colonizing plant tissues. Three to five days after the infection new sporangia begin to appear closing the life cycle of the pathogen. In the presence of opposing mating types, sexual reproduction can occur resulting in formation of oospores, structures capable of surviving winter and being a source of epidemics for the next year (Fry et al. 1993).

Characterization of *Phytophthora infestans*

Phenotypic markers

Several methods are currently used to characterize the genetic composition of *P. infestans* populations.

Niederhauser (1956) was the first to report the occurrence of sexual reproduction between two opposing mating types, proving the heterothallic nature of this species. Since then, the mating type test is widely used in characterization and monitoring of the pathogen (Goodwin et al. 1992b). A1 and A2 mating-type ratios are important to predict the extent of sexual recombination and the risk of the production of the persistent oospores serving as primary inoculum for the next cropping season. In addition to its epidemiological impact, sexual recombination is likely to increase the rate of gene flow between different pathogen populations and might result in increased aggressiveness in subsequent years.

Additional phenotypic markers like fungicide resistance to e.g. metalaxyl are also available to indicate the frequency of resistant genotypes present in populations and the usefulness of further application of fungicides (Dowley and O'Sullivan 1981).

Virulence tests described by Malcolmson and Black (1966) created an international late blight differential set. This consists of 11 potato clones, each containing a single resistance gene introgressed from the Mexican wild species *S. demissum* Lindl. This test is used to characterize the complexity of an unknown isolate by defining its virulence pattern.

DNA fingerprinting

An advance from phenotypic to genotypic methods of analysis was made thanks to molecular techniques and genome fingerprinting that generated novel DNA-based genetic markers. These markers allow tracking of the changes at the genomic level, with each marker system offering different possibilities adequate for various aspects of *P. infestans* research.

RG57

RFLP probe RG57 is used to investigate genetic diversity of *P. infestans* populations (Forbes et al. 1998; Purvis et al. 2001; Goodwin et al. 1992a; Cooke et al. 2003). It is a polymorphic probe that hybridizes to more than 25 randomly selected loci in the *P. infestans* genome. The genomic fragments hybridizing to RG57 show independent Mendelian inheritance and fragments do not appear to be allelic or closely linked with each other.

However, the method does have disadvantages like; large amounts of pure DNA are required, it is time-consuming and the banding patterns can be difficult to interpret.

SSR

SSR markers (simple sequence repeats) were developed for monitoring the genetic structure and comparisons of *P. infestans* populations (Knapova and Gisi 2002). This type of marker is also used for tracking particular strains, or monitoring inoculum movement.

SSRs have great potential to uniquely discriminate each strain because of their high degree of length polymorphism. SSR analysis focuses on few markers, but the precise nature of each locus and its length variation are unambiguously defined.

Both alleles at a locus are amplified and discriminated simultaneously, yielding co-dominant data appropriate for detailed population genetic analysis. The assay is PCR-based and only fraction of amount of DNA is required in contrast to RFLP. For analysis the DNA extracted from spores washed from a lesion or even a section of the infected leaf itself is adequate, obviating the need for pathogen isolation.

Mitochondrial DNA

Uniparentally inherited mitochondrial DNA markers are moderately conserved enabling tracking of specific lineages, providing a useful comparison to markers in the nuclear genome. Although it is a powerful tool, *P. infestans* mtDNA diversity is relatively limited. Carter et al. (1990) defined two mitochondrial types, type I and type II, by restriction of total DNA. Type II differed from type I by an insert of 1.6 kb and rearrangement of the flanking sequences. Type I was further differentiated into haplotypes Ia and Ib. Similarly, type II was subdivided into haplotypes IIa and IIb. There is little linkage of these markers to the fenotype, but for example, haplotype Ib has never become highly resistant to one of the most useful systemic and curative fungicides, metalaxyl.

Control of the disease

Soon after the "Irish famine", plant epidemiologists have investigated reproduction of the pathogen, between-season survival and ways to predict the effects of weather and input of surrounding vegetation into the overall late blight epidemic. This led to the commonly accepted sanitary precautions that help limit disease onset. They rely mainly on use of certified seed potato, elimination of potato refuse piles and volunteer plants that emerges in the following season. Agricultural practices like crop diversification, cultivar mixtures and mixing potato varieties with other crop species have also been tested (Andrivon et al. 2003; Pilet et al. 2006). In addition, organizations like Dacom Plant service B.V (http://www.dacom.nl) or Opticrop (http://www.opticrop.nl) help and advise the potato growers in forecasting and managing late blight threat by collecting and processing informations concerning late blight. However, still today the most efficient way of protection against this disease is the frequent applications of fungicides, but increased aggressiveness of *P. infestans* observed in the early 80's became troublesome for potato growers since highly resistant strains to fungicides arose (Dowley and O'Sullivan 1981).

Renewed interest in *R*-gene based resistance as a possibility of crop protection is currently again investigated. This resulted in identification of sources of resistance in several wild *Solanum* species.

Resistance genes

Plants are constantly exposed to various pathogens in nature and some protective layers that render them resistant have evolved. Besides physical barriers, plant immune system is based on extracellular trans-membrane receptors detecting a wide range of conserved microbe-associated molecules. The second layer is effective against specialized pathogens that can successfully break through the first one and is based on resistance genes (Takken and Tameling 2009). Resistance responses to pathogens are traditionally classified as race-specific, race-nonspecific, and non-host resistance. Over 55 *R*-genes have been cloned in the past two decades from a wide variety of plant species, mostly from Solanaceae. The plant *R*-genes products encode proteins that share a limited amount of conserved elements on basis of which they were divided into several classes. The majority belongs to the nucleotide binding site and leucine-rich repeats (NBS-LRRs). Relatively fewer are acting as a kinase or extracellular receptor with extracellural LRR connected to variable cytoplasmic region of the protein (Van Ooijen et al. 2007).

***Solanum* - sources of genetic resistance**

Disease resistance in plants might occur at different levels: subspecific or varietal or at the species or genus level (non-host resistance). In addition, resistance is quantitative (horizontal) reducing disease severity or race or cultivar-specific mediated by single resistance (*R*) genes (vertical). Resistance to late blight in potatoes was first observed in 1845-1847 when some of the genotypes survived despite the severe epidemic of late blight. At the beginning of the 20th century researchers discovered that many wild *Solanum* species contain resistance genes (Jansky 2001). Having huge diversity available (Hawkes 1990), numerous genes conferring vertical and horizontal resistance have been identified, promising improvement of crop quality (Gebhardt and Valkonen 2001).

Race-specific *R*-genes

Race-specific resistance, also called qualitative or vertical resistance, is based on the presence of major resistance *R*-genes which are simply inherited in accordance with Mendel's laws. Race-specific resistance is only effective against certain strains of the pathogen, and is easily overcome by rapid evolution of the pathogen, resulting in a lack of durability in the field. *Solanum demissum* was the main source of dominant resistance genes introgressed into commercial cultivars (Malcolmson and Black 1966; Mastenbroek 1953).

By introgression breeding eleven *R*-genes against late blight were found and some crossed in up to the cultivar level. However the durability of the genes appeared to be short, since virulent races of the pathogen appeared quickly in cropping seasons following market introduction. Still, by the mid 20th century, late blight was kept at a tolerable level mainly by agricultural practices and the use of moderately resistant cultivars (Fry and Goodwin, 1997).

So far eight R-genes of *S. demissum* have been localized on the genetic map of potato: *R1*, *R2*, *R3a/b*, *R6*, *R7*, *R10*, *R11* (Leonards-Schippers et al. 1992; Li et al. 1998; El-Kharbotly et al. 1994, 1996; Huang et al. 2004; Bradshaw et al. 2006) and R1 and R3a/b have been isolated (Ballvora et al. 2002; Huang et al. 2005a). However compatible pathogen races to these *R*-genes are currently commonly present in the *P. infestans* population making these *R*-genes useless in agronomic sense.

The other source of monogenic resistance has been identified on chromosome 7 of *S. pinnatisectum* Dunal species by Kuhl (2001), but still it is of question whether this identified gene is a novel gene or R9 like gene. Also *S. berthaultii* Hawkes might carry substantial amount of genes as presented by Ewing et al. (2000).

Race non-specific *R*-genes

By contrast to the race specific resistance, race-nonspecific resistance is effective against all known strains or races of a pathogen and is generally of a partial nature. This resistance manifests itself by a reduction of infection rate and can be found in several *Solanum* species (Ross 1986). This type of resistance is also known as polygenic, general, or quantitative because it depends on many genes, each having some additive effect to resistance. Genetic components controlling race non-specific resistance appear to be located on almost all potato chromosomes (Gebhardt and Valkonen 2001). This type of resistance does not confer complete resistance, but rather slows down the rate of the disease. It is believed that this type of resistance is more durable. The partial resistance of a non-race specific nature, found in *S. tuberosum* cultivars such as 'Surprise' and 'Pimpernel', has lost nothing of its effectiveness for more than 30 years since the introduction in 1950's (Colon et al 1995). However, the resistance is not strong enough to give sufficient protection. Furthermore, a strong association exists between partial resistance and late maturity, which are undesirable traits in potato production.

There is also strong indication that field resistance is associated with late maturity. Partial resistance is also present in a number of wild *Solanum* species (Hoekstra and Seidewitz, 1987).

Only recently molecular markers made possible the estimation of phenotypic values and the tracking of quantitative genes. *Solanum bulbocastanum* Dunal, a diploid Mexican species, potentially offers this type of resistance (Niederhauser and Mills 1953). This new source has been exploited and until now three *R*-genes have been discovered and isolated from this species: *RB/Rpi-blb1* (Song et al. 2003; Van der Vossen et al. 2003), *Rpi-blb2* (Van der Vossen et al. 2005) and *Rpi-blb3* (Naess et al. 2000; Van der Vossen et al. 2003; Park et al. 2005).

Despite its sexual incompatibility with cultivated potato, resistance genes have been introgressed successfully from *S. bulbocastanum* to cultivars through interspecific bridge crosses. In case of *Rpi-blb1*, for more than 20 years of breeding procedures that included yearly field tests no compatible strains of *P. infestans* were detected. This created a huge expectation that this gene will be a durable one with value for future breeding purposes. It was believed that late blight resistance in *S. bulbocastanum* had arisen early in evolution and could detect proteins of *P. infestans* crucial during early stages of infection, thus it is not likely that mutation that might lead to overcome this gene can arise easily. However, only recently compatible strains of *P. infestans* have been detected diminishing the enthusiasm (Fry 2008). Still it is possible that a polycluture of *S. bulbocastanum R*-genes might lead in the future to cultivars free of disease.

Nowadays also in other wild *Solanum* species the presence of QTL's associated to *R*-genes have been detected and described (Meyer et al. 1998; Collins et al. 1999; Sandbrink et al. 2000; Ghislain et al. 2001; Visker et al. 2003; Villamon et al. 2005, Smilde et al. 2005; Sliwka et al. 2006).

Non host resistance

Non host resistance is the most common form of disease resistance exhibited by plants. This type of resistance is expressed toward the majority of potentially pathogenic organisms and is thought to comprise a variety of distinct mechanisms. They may include the presence of barriers, production of toxins, lack of essential metabolites or signaling molecules required by the pathogen. Perhaps, non host resistance is mediated by a set of ancient broad-spectrum *R*-genes that coevolved with pathogen populations following the trench-warfare model. In this scenario, a non host interaction takes place when a high frequency of *R*-gene alleles in a plant species is combined with a high frequency of the matching *Avr*-gene alleles in the pathogen.

Until now this type of resistance has received little or no attention due to difficulties in obtaining tractable genetic systems.

However, recent progress in genetic engineering and development of virus induced gene silencing may help us to identify factors responsible for this type of resistance.

Screening methods available for resistance screening and race structure determination

To be able to exploit wild sources of resistance a series of methods have been described to assess foliar late blight resistance. Beside field tests and whole plant greenhouse assays (Stewart et al. 1983), laboratory tests on detached leaves (Lapwood 1961; Vleeshouwers et al. 1999), leaflets (Malcolmson 1969), leaf discs (Hodgson 1961) or in vitro plants (Huang et al. 2005b) were developed to identify valuable genotypes resistant to *P. infestans*.

Field tests

Field tests described by Fry (1978) and Colon and Budding (1988) closely resemble the optimal conditions under which late blight disease develops. This is a highly accurate test when performed under optimal conditions, both for the pathogen and the host plant. Even discrete differences in disease response can be measured, and simultaneously evaluation of the function of *R*-genes in field conditions can be performed. Obvious disadvantage is that field testing can only be performed once a year during the growing season and additionally weather conditions can significantly influence the result. Resistance assessment in the field is expressed as AUDPC values (Area Under the Disease Progress Curve) (Shaner and Finney 1977), which are considered the best estimates of disease for multi-cycle pathogens like *P. infestans* (Fry 1978).

Whole plant greenhouse assays

The whole-plant assay (Stewart et al. 1983), used to screen for late blight resistance is done in greenhouse facilities under greenhouse conditions. Replicates of clonally propagated plants are arranged randomly and spraying is done with a fine mist of *P. infestans* sporangial suspension. Relative humidity and the temperature in the greenhouse can easily be maintained at optimal level making this test independent from an outside environment. The blight scale, with 0 indicating a fully blighted plant and 9 indicating no visible infection, is often used to visually rate disease severity.

Detached leaf assays

The detached leaf assay is a laboratory assay performed on 4-6 weeks old plants (Vleeshouwers 1999). Sporangial suspension of the isolates is prepared from a 2-3 weeks old culture grown on 10% agar, or alternatively the sporangium suspension can be obtained by rinsing leaves of a susceptible cultivar previously infected with *P. infestans*. Incubation of infected plant material takes place in climate chambers where optimal conditions for pathogen growth (temperature, light, humidity) are maintained. In the laboratory test, a commonly used parameter for resistance assessment is lesion growth rate (LGR). Because of its great efficiency, minimal space required, simplicity and possibilities of simultaneous screening of hundreds of genotypes in a short time, this is nowadays the most often used assay for phenotype.

In vitro plants assay

In vitro assay combines the advantage of using whole, healthy and uniform plantlets with minimal space required for the test. Additionally an experiment can be planned with flexibility because of the direct availability of the plants. Huge drawback is that no quantitative aspect of the disease can be tested and it is laborious to bring plants into the in vitro conditions (Huang et al. 2005b).

Effector screening

To colonize host tissues, the plant pathogen secretes effector proteins (Avr proteins) that modulate host innate immunity and enable parasitic infection (Kamoun 2007). Recent progress in sequencing the *P. infestans* genome and cloning of a few oomycete avirulence genes made it possible, based on conserved sequence fragments (RXLR motif) to predict existence of several candidates avirulence genes. This approach promises the acceleration of identification and functional characterization, of broad-spectrum *R*-genes against late blight, enabling faster engineering of more durable late blight resistance into potato varieties (Vleeshouwers et al. 2008).

Scope of the thesis

The present PhD project is part of the potato program carried out within the Centre for Biosystems Genomics (CBSG). The CBSG is a network of Dutch scientists in the field of plant genomics, as well as Dutch companies involved in plant genetics, breeding, cultivation and processing.

The objectives of the work carried out and described here was to gain knowledge about *S. dulcamara*, one of few native *Solanum* species present in Europe, and its resistance to *P. infestans*. In chapter 2 I present molecular data that were generated on the accessions that represent a cross section of European accessions of this species. I graphically presented genetic variation between and within the accessions that were collected in various places and habitats across Europe.

In chapter 3 I describe observations of late blight occurrence on *S. dulcamara* plants growing in natural habitats across The Netherlands and in conditions of experimental field where plants were infected artificially. Chapter 4 presents identification and mapping of *Rpi-dlc1* – the first resistance gene against *P. infestans* identified in *S. dulcamara*. Additionally chromosome 9 where *Rpi-dlc1* was mapped was compared with potato mapping population and available potato/tomato genetic maps. A possibility for presence of additional resistance genes in *S. dulcamara* was concluded after testing with additional set of *P. infestans* isolates. Chapter 5 describes the identification of a second resistance gene to late blight identified in *S. dulcamara*, named *Rpi-dlc2*. Additive effect of *Rpi-dlc1* and *Rpi-dlc2* on the level of resistance was shown in a field experiment using the population containing the two identified genes. In chapter 6 I discuss problems encountered during the experimental work, main breakthroughs and possible applications of results. Some future perspectives concerning *S. dulcamara* research are also discussed.

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Genetic structure of European accessions of *Solanum dulcamara* L. (Solanaceae)

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Abstract. *Solanum dulcamara* or bittersweet is one of the few native species of *Solanum* present in Europe. It is a common weed that occupies a wide range of habitats and is often found in direct vicinity of cultivated potato (*Solanum tuberosum*) where it can transmit diseases. A broad sampling of European *S. dulcamara* accessions was carried out to gain insight into the population structure and crossing preferences of this species. Three AFLP primer combinations generating 288 polymorphic fragments were used to analyze 79 bittersweet accessions (245 individuals). Dendrograms revealed a low level of genetic polymorphism in the bittersweet populations, caused partially by the outcrossing nature of this species.

Solanum dulcamara L., commonly named bittersweet or climbing nightshade, is one of the few native species of *Solanum* L. in Europe. It is a well known host for the potato quarantine pathogen *Pseudomonas solanacearum* (Smith) Smith a causal agent of bacterial wilt or brown rot (Olsson 1976; Elphinstone et al. 1996; Janse 1996), and may play an important role in potato late blight epidemiology (Flier et al. 2003; Dandurand et al. 2006). In some countries in North-West Europe where potato is cultivated, for these reasons, *S. dulcamara* was subjected to eradication programs from the natural vegetation, aimed at preventing spread of *P. solanacearum*. *Solanum dulcamara* is one of the about 1500 species of the genus *Solanum* (Weese and Bohs 2007), a genus with a cosmopolitan distribution (D'Arcy 1991). *Solanum dulcamara* belongs to section *Dulcamara* (Moench) Dumort. of subgenus *Potatoe* (G. Don) D'Arcy (Child and Lester 2001). Subgenus *Potatoe* also includes the cultivated potato and its wild relatives. Section *Dulcamara* has been studied by several authors and there is no consensus on the size and content of the section. Child and Lester (2001) recognized three sections in the Dulcamaroid taxa: section *Jasminosolanum* Bitter ex Seithe, section *Californisolanum* A. Child, and section *Dulcamara*, the latter containing four to five Eurasian species. Recent analyses of the *ndhF*, *trnT-F* and *waxy* genes (Bohs 2005; Weese and Bohs 2007) showed that the Dulcamaroid clade, including *S. dulcamara*, clusters closely to a Morelloid clade (containing the species of *Solanum* section *Solanum*). *Solanum dulcamara* is a species widely naturalized in the entire holarctic area (Horvath et al. 1977). This diploid (2n=24), perennial plant occurs in contrasting environments, from wet habitats of irrigation ditches, river banks and lake shores to dry areas of dunes and plains.

Seeds are mainly dispersed by birds. Fruits as well as vegetative parts of the plant can be transported by water. The present study was conceived to gain information on the infraspecific variation within *S. dulcamara*. We used the collection in which geographical provenance and provenance from different habitats of the various accessions could cover a broad range of genotypic variation in Europe as well as a wide spectrum of *R* genes.

Furthermore, to better understand the mechanism of gene flow between *S. dulcamara* populations, we established the breeding system of this species in its natural environment.

Materials and methods

Plant material

Seeds of *S. dulcamara* from the Solanaceae collection of the Radboud University Botanical and Experimental Garden, Nijmegen (The Netherlands) were used. In addition we used seeds and cuttings collected in 2005 and 2006 from *S. dulcamara* plants in their natural habitats.

The accession numbers are abbreviated e.g.: accession number 934750233 is abbreviated to 93233 or A54750207 to A5207. Passport data concerning the accessions used can be found at http://www.ru.nl/bgard. All plants were cultivated at the Radboud University Botanical and Experimental Garden. Plants were grown in the greenhouse under long day conditions (16hrs day / 8 hrs night). Supplementary light was given by using high pressure sodium lamps (SON-T 600 W). Plants were grown in pots filled with a standard soil mixture (Lentse Potgrond no.4). Regularly, the plants were fertilized with Kristallon Blauw (Yara Benelux B.V. Vlaardingen).

Genomic DNA isolation

Total genomic DNA was isolated from young leaves using the Wizard genomic DNA purification kit (Promega), according to the protocol supplied by the manufacturer. A pestle was used to grind 40mg of fresh plant material in liquid nitrogen to a fine powder. The concentration of DNA was assessed using a spectrophotometer (Pharmacia Biotech: GeneQuant II) and the quality of the DNA was checked by electrophoresis in a 1% agarose gel stained with Ethidium bromide (Eurogentec, Belgium).

Genomic profiling

The AFLP® method was used to estimate genetic distances among *S. dulcamara* accessions. AFLP was performed according to Vos et al. (1995) with minor modifications. 0.5 µg DNA was digested with *Eco*RI and *Mse*I restriction enzymes (Fermentas, Germany). After ligation and pre-amplification, selective amplification was performed using D4 dye (Beckman Coulter) with labeled EcoRI primer together with unlabelled MseI primer using thermocycler GeneAmp9600 (Perkin Elmer, USA). Three primer combinations were used each with three selective nucleotides; EcoRI+AAC/MseI+CAC, EcoRI+AAC/MseI+CAT and EcoRI+ACC/MseI+CAT.

Table 1. Accessions used to test genetic distances among accessions of *Solanum dulcamara.*
The first column refers to Figure 1 and indicates the place where accessions were collected The second column is an abbreviated accession code. The third column indicates the habitat of each accession. The fourth column indicates how many individuals were used in the experiment. In the dendrogram letters of alphabet indicate individual genotype e.g. A5/067c. C refers to third individual in A5067 accession. Outgroup species are not included.

Map nr	Accession code	Habitat	Individuals used	Map nr	Accession code	Habitat	Individuals used	Map nr	Accession code	Habitat	Individuals used
1	93233	aquatic	4	28	A5249	aquatic	3	54	A5234	aquatic	4
2	A5005	aquatic	4	29	A5250	aquatic	2	55	A5235	aquatic	2
3	A5007	aquatic	4	30	A5251	dunes	3	56	A5236	dry	3
4	A5008	aquatic	4	31	A5253	dunes	4	57	A5237	unknown	3
5	A5009	aquatic	2	32	A5254	dunes	4	58	A5238	unknown	4
6	A5101	aquatic	1	33	85003	unknown	3	59	A5239	unknown	4
7	A5102	aquatic	1	34	88034	unknown	3	60	A5242	aquatic	4
8	A5170	aquatic	1	35	88057	unknown	2	61	94001	unknown	3
9	A5171	dry	1	36	91008	unknown	4	62	A4147	unknown	4
10	A5172	plains	1	37	91046	unknown	4	63	A4148	unknown	4
11	A5173	plains	4	38	91081	unknown	3	64	A4149	unknown	4
12	A5174	dunes	4	39	92023	unknown	4	65	A4151	unknown	4
13	A5175	dunes	1	40	92109	unknown	4	66	A5002	unknown	4
14	A5176	dunes	4	41	92195	unknown	4	67	A5066	aquatic	1
15	A5177	dunes	4	42	A5191	dry	4	68	A5067	aquatic	4
16	A5178	dunes	3	43	A5192	dry	4	69	A5068	aquatic	1
17	A5179	dunes	4	44	A5196	aquatic	1	70	A5069	aquatic	4
18	A5185	aquatic	3	45	A5197	unknown	3	71	A5180	dry	4
19	A5187	aquatic	1	46	A5198	aquatic	3	72	A5181	aquatic	3
20	A5188	aquatic	4	47	A5200	aquatic	4	73	A5183	aquatic	4
21	A5189	aquatic	3	48	A5201	aquatic	4	74	A5184	aquatic	3
22	A5243	dry	2	49	A5202	unknown	1	75	A5194	unknown	4
23	A5244	aquatic	4	50	A5204	unknown	2	76	A5195	unknown	1
24	A5245	dry	4	51	A5205	aquatic	4	77	A5203	dry	1
25	A5246	aquatic	4	52	A5206	unknown	2	78	A5241	aquatic	3
26	A5247	aquatic	4	53	A5207	aquatic	3	79	A5063	unknown	4
27	A5248	aquatic	3								

PCR products after selective amplification were diluted 10 times in Sample Loading Solution (SLS Beckman Coulter). Two µl of this dilution were added to 38 µl of SLS containing 0.2 µl of CEQ DNA size standard 600 (Beckman Coulter).

The fragments were analyzed using a Beckman Coulter 8000™ fragment analysis system with default values of study parameters, with exception of size standard (600) and model of study (cubic). To investigate the breeding system of the species occurring in natural conditions, the AFLP method was performed as described above, but for selective amplification EcoRI primer labeled with γ33P (mp Biomedicals, USA) was used. Two primer combinations each with three selective nucleotides; EcoRI+AAA/MseI+CCC, and EcoRI+AAC/MseI+CAA were used. Selective PCR products were separated on 5% polyacrylamide gel (Duchefa, The Netherlands), dried on paper and visualized by exposure to X-ray film (Kodak BIOMAX MR) for 48 hours. The generated radiograms were scored manually, which was feasible due to the lower number of markers.

Genetic distances among accessions of *S. dulcamara*.

245 individuals (79 accessions) of *S. dulcamara* (Table 1, Figure 1) were examined. For comparison, three accessions of *Solanum* species belonging to section *Solanum* were included (*S. americanum* Mill., *S. villosum* Mill., *S. nigrum* L.). Combined results from three AFLP primer combinations generated the data matrix from which monomorphic and single fragments (present only in one individual) were removed, creating a dataset containing 288 characters. This dataset was analyzed in NTSYS-pc® version 2.11T (Rohlf 2000). The SimQual program for qualitative data with both the Simple Matching (SM) and Jaccard (J) coefficients were used to generate similarity matrices. Clustering was performed using the SAHN option and the unweighted pair-group method (UPGMA).

Figure 1. Graphical representation of the accessions used to test genetic distances among European accessions of *Solanum dulcamara*. Numbers refer to the map nr. listed in table 1.

Estimation of the breeding system of *S. dulcamara* occurring in its natural environment

Three different mother plants were collected together with their respective mature berries in Castricum aan Zee, Egmond aan Zee and Uitgeest (The Netherlands).

Cuttings were rooted and maintained, and seeds were extracted. Eight seeds per mother genotype were sown. DNA of the three mother plants and eight offspring genotypes were compared using AFLP.

Results

Genetic distances among accessions of *S. dulcamara*

The purpose of this experiment was to gain insight into the population structure of *S. dulcamara*. We examined the level of genetic variation within and among 79 bittersweet accessions growing in various habitats and different geographical regions, from all over Europe, with an emphasis on Dutch material (51 accessions). We used SM and J coefficients to graphically visualize genetic distances between accessions of this species. The generated dendrograms were compared with each other and no important differences were found (only small differences occurred in the clustering of single individuals). For further analyses we focused on the SM dendrogram (Fig. 2). Four major clusters (Fig. 2; Cluster I, II, III, IV) could be recognized, within which several smaller groups have been distinguished. There are two groups where no clear structure is present and mixing of individuals from The Netherlands and the rest of Europe occurs (Fig. 2; Group A, B). In those regions only individuals from two accessions interrupt the random pattern by grouping together: one from Spain and one from The Netherlands in group B, and five individuals grouped together from four Polish accessions in group A. Cluster I contains only genotypes collected outside of The Netherlands. This cluster is well structured and all the individuals from an accession are grouped together (with the exception of accession A5/067cGB). The geographical distance and genetic differences between accessions are reflected in their actual position in the dendrogram. Accessions of Italian origin are genetically more similar to the French and German accessions than to accessions originating from north Europe, particularly from Belgium, United Kingdom, The Netherlands and Poland. Genotypes of Dutch and Polish origin are not represented. Cluster II contains accessions from almost entire Europe. This cluster is divided into three subgroups (1, 2, 3). The first subgroup (1) contains accessions that were collected outside The Netherlands (exception: A5/102) and consists of individuals that derived from seeds that had been collected many years before this study was carried out. In this group, clustering of similar genotypes does occur, but is rare and does not reflect the geographical distance between accessions. The second subgroup (2) is restricted to genotypes collected in Poland (exception: A5/066, A5/202).

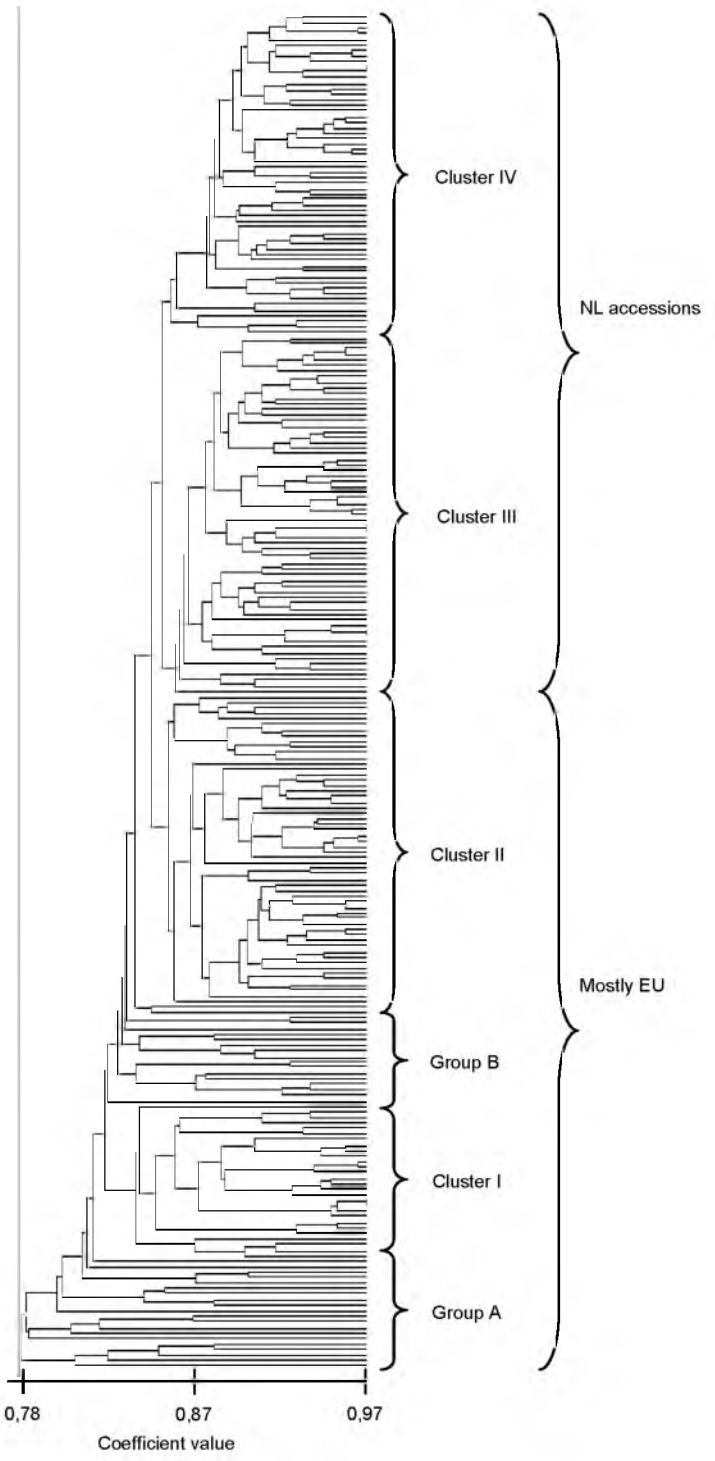

Figure 2. Overview of the simple matching (SM) dendrogram based on 288 polymorphic AFLP fragments of European *Solanum dulcamara* accessions, showing the four main clusters (I, II, III, IV) and two groups (A, B) identified in this study. **Figure 2a** and **2b** show enlargements of four main clusters with the subgroups identified within them. NL – The Netherlands, FR – France, BE – Belgium, GB – Great Britain, IT – Italy, DE – Germany, PL – Poland, AU – Austria, SW – Switzerland, SP – Spain.


Cluster II
Cluster I
3
2
1
Group B
Group A
A5238dNL
A5195lT
A5002dlT
A5002blT
A5002clT
A5238cNL
A5238bNL
A5238aNL
A5201dNL
A5201bNL
A5201cNL
A5201aNL
A5202NL
A5184bPL
A5184aPL
A4149bPL
92195dPL
A4147aPL
A4147bPL
92195cPL
92195aPL
A4151dPL
A4151cPL
A4147dPL
A4147cPL
A4149dPL
A4149cPL
A4148aPL
A4148dPL
A4148bPL
A5066GB
92109aFR
92109dFR
91081bFR
88034bAU
85003cDE
85003aDE
92023cBE
92023aBE
91081cFR
92023bBE
91008bSW
91008cSW
91008dSW
91046clT
91046alT
A5102NL
91008aSW
A5239cNL
A5007bNL
A5236bNL
A5236cNL
A5187NL
A5206bNL
A5007aNL
A5197cNL
A5197bNL
A5197aNL
A5245cNL
A5245dNL
A5007cNL
A5241cSP
A5241aSP
A5241bSP
A5184cPL
A5203FR
A5194cBE
A5194dBE
A5194aBE
A5068GB
A5067cGB
A5183dlT
A5183clT
A5183blT
A5183alT
A5181clT
A5181blT
A5181alT
A5063dFR
A5063bFR
A5063cFR
A5063aFR
A5180dDE
A5180aDE
A5180cDE
A5180bDE
A5067dGB
A5067aGB
A5067bGB
A5069dGB
A5069cGB
A5069bGB
A5069aGB
A5254bNL
A5006cNL
A4151bPL
A4151aPL
A4148cPL
A4149aPL
92109bFR
98001bGB
98046dlT
98001cGB
A5236aNL
A5178cNL
A5002alT
92195bPL
A5234dNL
A5254cNL
98233dNL
A5194bBE
A5244aNL

Figure 2a

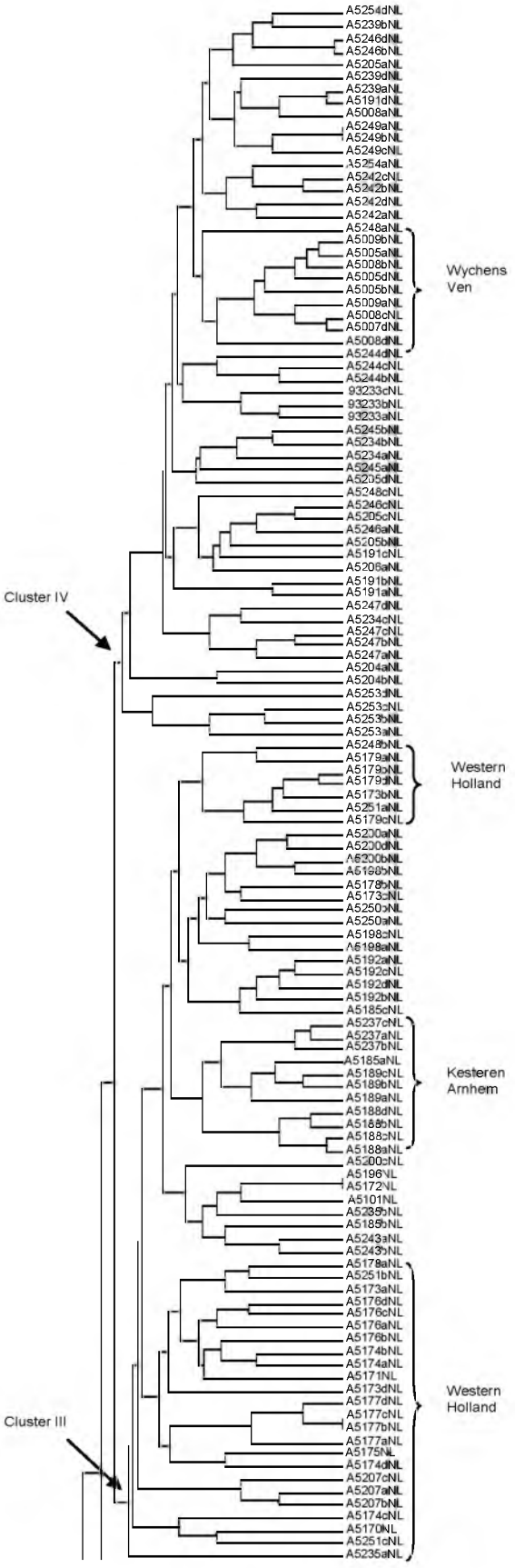
Cluster IV
Cluster III
Wychens Ven
Western Holland
Kesteren Arnhem
Western Holland

Figure 2b

The third subgroup (3) includes two accessions from The Netherlands (collected in the Flevoland district area) and two accessions of Italian origin.
Cluster III consists of only Dutch accessions. This cluster shows partial structure and clustering of individuals from the same accession does occur. Further two branches containing genotypes collected from the dune areas of western Holland are distinguished. The smaller branch contains 5 and the larger 16 individuals out of the 31 used in this study. Also plants collected on the banks of the Nederrijn River (Kesteren) and in Arnhem form a group of 11 plants out of the 22 used. However, in both cases individuals representing those groups are also present in other parts of the dendrogram and in the fourth cluster.
Cluster IV shows a high homogeneity among the rest of the Dutch accessions. Clustering of the individuals from the same accession is present, but it is rarely linked with a particular region of sampling. Only accessions from the Wychens Ven area form a separate group, but not all of the genotypes representing this region are clustered. Some are present in different regions of the dendrogram.

Estimation of the breeding system of *S. dulcamara* occurring in natural environments

This experiment was carried out to test whether naturally growing *S. dulcamara* is an out- or in-breeding species. The AFLP results showed that the majority of the offspring contained AFLP fragments that are not present in the mother genotype. In total 24 offspring plants were tested (eight from each parent) and only two plants collected in the region of Egmond aan Zee showed a banding pattern identical to the mother genotype (AFLP data not shown).

Discussion

The aims of this study were to investigate the genetic structure of European bittersweet and the in- or out crossing nature of the species. We analyzed accessions collected at various places and habitats throughout Europe to include as much variation as possible. In these experiments AFLP markers were used because of their many advantages for this type of study (Mueller and Wolfenbarger, 1999). AFLP generates a great number of neutral characters providing high resolution in studies aiming to compare closely related individuals.
The experiment on the genetic distances among accessions of *S. dulcamara* was designed to gain insight into the genetic structure of wild populations, and to see whether cluster analysis reveals any correlation between genomic similarity and geographical provenance of the populations. The average similarity in AFLP patterns of widely sampled bittersweet populations proved to be high (>80%; Fig. 2). Extremely high genetic similarity between populations of wild *Solanum* species was reported earlier for the *S. nigrum*.
Scholte (2000), detected genetic similarity above 94.7%, but in that case the author was dealing with a polyploid and selfing species, where homogeneity is expected.

As investigated here, the breeding system of naturally growing *S. dulcamara* plants indicates that it is a cross pollinating species. We have found only two individuals that could be a result of self fertilization. However, for *S. dulcamara*, being a diploid out crossing species, the high similarity found was a surprise. We discuss two possible explanations for the homogenity encountered. Firstly, bittersweet is a very common plant, at least in The Netherlands. It has an efficient reproduction, both vegetatively and generatively. Also, the seeds are easily dispersed by birds or water. This promotes a high gene flow between populations, increasing uniformity. Secondly, in contrast to *S. nigrum*, bittersweet is a perennial plant that can contribute seeds to several generations. In *Populus nigra* L. (Arens et al. 1998) it was found that the longevity of the plant made crosses between generations possible, increasing the similarity within the population (82.7%). The same could be the case in *S. dulcamara*.

The genetic similarity between accessions collected outside The Netherlands (Fig. 2 cluster I, II, group A, B) is slightly lower than the similarity among the Dutch accessions, reflecting the larger geographical separation of the genotypes used. The similarity among the individuals within cluster I and cluster II accessions is comparable to that among the material collected in The Netherlands (cluster III, IV).

We detected only partial clustering by habitat or geographical origin of the accessions. The European accessions in cluster I are arranged according to country of origin, but in cluster II representatives from different countries are mixed, except the Polish. An explanation for that situation might be that those genotypes were derived from various botanical gardens and were propagated for a number of years, during which period uncontrolled crosses could have taken place.

The clustering of individuals originating from the same country as seen in cluster I and the partial clustering according to the habitat or region as seen in cluster III and IV suggest that there is some level of genetic variation present in the species, but it is only apparent when a few individuals from the separated populations are compared. Differences are fading when a large population from the same area is compared (like the other Dutch accessions in cluster III and IV). There the individuals from the same accession cluster, but rarely together, indicating that accessions from local areas are not genetically significantly different from each other.

If individuals from same region of sampling group together, very often few individuals from that region are scattered or form small groups in parts of the tree that contains genotypes from other areas. This is the case for the Wychens Ven population (Fig. 2; Group A and B; Cluster IV) where in cluster IV, 9 out of 10 individuals are grouped. Other 4 genotypes of this population are in Group A and B, one individual in cluster IV, but separated from the Wijchens Ven accessions. The same can be true for the population of western Holland with a main group (lower part of cluster III) and a small group (upper part of cluster III).

Table 1 lists the habitats of the accessions studied (aquatic, dry, dunes, plains).
Within the Dutch accessions, there is a certain clustering of populations originating from dunes, rivers and the Wychens Ven area. This could indicate that genotypes with certain ecological preferences are more similar to each other. However, Pegtel (1985) studied the germination percentage of two *S. dulcamara* accessions from wet and dry habitats at six constant and five diurnal temperature fluctuations and found little or no evidence of major variability's in behavior between the two populations.
Still, the differences could be masked by environmental influences. Also Clough et al. (1979) showed that there was no ecotypic differentiation with respect to light and shade habitats within *S. dulcamara*.
In previous studies authors described a morphological variation between *S. dulcamara* populations; like variable density in hairiness of the leaves (Horvath et al. 1977), erect or procumbent form of growth (van Ooststroom and Reichgelt 1966), described both in natural conditions and in the experimental plots. Apparent morphological differences concern: hairy or non hairy stem and leaves, different length/breadth ratios and shapes of leaf, differences in the way the inflorescence is formed and in the number of flowers per inflorescence (own observations). There is also a great variation in shapes and sizes of flowers and berries. However, we did not find any correlation between groups of similar phenotypes and groups identified in the AFLP dendrogram. It is possible that morphological traits are inherited by a relatively low/high number of genes, thus having little or no influence for the overall structure of the dendrogram. Similar conclusions were drawn by Dehmer and Hammer (2004) who investigated *S. nigrum* and *S. villosum* species, finding no correlation between morphology-based and the AFLP-based phenograms.
It is concluded that European *S. dulcamara* is a solid, easy to recognize out-crossing species that shows a rather faint tendency to sub-cluster according to geographical derivation or ecological niches. The genetic similarity between individuals within and between accessions is high. Apparently, this does not interfere with the species capacity to adapt to various contrasting habitats such as dry dune areas and wet lands where, at least in The Netherlands, it grows abundantly between common reed (*Phragmites australis* (Cav.) Steud.).
Despite the broad occurrence of bittersweet in many European countries and its possible impact as a source of *Phythophthora* in cultivated potato, (Platt 1999; Cooke et al. 2002; Flier et al. 2003; Dandurand et al. 2006) our knowledge about this species is very limited. Only a few experiments were done aimed at answering different research questions (Hare 1983; Wang et al. 1994). Up till now we were lacking insight in the amount of genetic diversity present in this weedy species. The present study provides this insight.

The authors acknowledge CBSG for funding this project. Thanks also go the gardeners of Radboud University Botanical Garden and Experimental Garden for their help with maintenance of *S. dulcamara* plants.

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3

Role of *Solanum dulcamara* L. in potato late blight epidemiology

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Abstract

Four sites with naturally growing *S. dulcamara* were surveyed during the 2006 and 2007 for the presence of late blight. Despite the two years of observations no late blight was detected among natural populations of bittersweet. Instead, repeated infections occurred on few *S. dulcamara* plants from a collection growing in botanical garden. These plants were used to investigate the possibility of survival of the inoculum between seasons. At mean time, a set of 21 and 52 *S. dulcamara* accessions inoculated with *P. infestans* under field conditions resulted in a wide range of responses to the disease. More susceptible reactions were found among genotypes collected at greater distance from commercial potato fields indicating the possibility of genetic selection caused by *P. infestans*. However, both, scarceness of natural infections and no overwintering indicated that bittersweet may not play a role in late blight epidemiology.

Introduction

The oomycete *Phytophthora infestans* (Mont.) de Bary is the most important disease of cultivated potato (*Solanum tuberosum* L.) (Fry 2008). To limit the chance for disease onset, management strategies in potato production rely mainly on using certified seed material and reduction of the sources of primary infections. This is done by covering potato dumps and destroying volunteer plants that may emerge from tubers surviving the winter period and that may carry inoculum from the previous cropping season. Further, frequent preventive spraying with fungicides, between 10-16 times per season in The Netherlands, is a requirement in the disease management. *Phytophthora infestans* is able to infect other solanaceous species present in nature. The role of these plants as an alternative source of primary infections or as a source of sustaining late blight pressure has been considered, but it has not been fully confirmed up to now. The studies thus far have been based either on (i) monitoring of weedy plants in their natural vegetation for disease symptoms or by (ii) artificial inoculations of potential hosts under laboratory conditions. An early observation of natural infection by *P. infestans* of weedy Solanaceae in Europe was described by Hirst and Steadman (1960) with late blight occurring on *S. nigrum* L and *S. dulcamara* L. More recent reports of infections are from 1999 and 2001 described by Flier et al. (2003a) and Deahl et al. (2004). Beside *S. nigrum* and *S. dulcamara*, four other species of Solanaceae: *S. physalifolium* Rusby, *S. sarrachoides* Mill., *S. villosum* Lam., and *S. sisymbriifolium* Lam. (the latter species used as a trap crop for sanitation of fields infected by potato cyst nematode) are present in Europe and United States and may serve as an alternative host. There are reports of late blight being identified in the vicinity of potato fields in early summer of 2003 on *S. physalifolium* (Andersson and Johansson, 2003), on *S. sisymbriifolium* in 2000 and 2001 (Flier et al. 2003a) and *S. sarrachoides*.

The latter species, also known as hairy nightshade, was found infected in North America, where it is a common weed present on arable fields (Olanya et al. 2005). Other cultivated Solanaceae such as tomato (*S. lycopersicum* Mill.) and to some extent petunia (*Petunia hybrida* Vilm.) (Deahl and Fravel 2003), *S. scabrum* Mill. (Fontem et al. 2004) and *S. sisiymbriifolium* (Flier et al. 2003a) are found infected by *P. infestans*.

To prove the role of wild solanaceaous species as an alternative host for late blight, it has been also attempted to confirm pathogenicity of *P. infestans* on wild hosts under laboratory conditions (Peterson 1947; Platt 1999; Cooke et al. 2002; Andersson and Johansson 2003; Deahl et al. 2004; Fontem et al. 2004; Olanya et al. 2005; Lebecka 2007).

In The Netherlands, two solanaceous species are very common: *S. nigrum* that grows as an annual weed on arable land and *S. dulcamara*, a perennial plant present abundantly along canals, in wetlands, irrigation ditches and less frequent in dune areas along the North Sea coast. *Solanum dulcamara*, commonly named bittersweet or climbing nightshade, has been known for a long time as a host for *P. infestans* under natural conditions (de Bary 1876; Cooke et al. 2002; Flier et al. 2003a). However, the potential role of bittersweet as reservoir of inoculum, from which epidemics in commercial potato production might begin, has not been proven thus far.

In the framework of a broader study, aiming at understanding how wild solanaceous species cope with the occasional extremely high late blight pressure during the growing seasons in The Netherlands, surveys were carried out to estimate frequency of natural late blight infections occurring on *S. dulcamara*. To investigate the role of *S. dulcamara* as a host for *P. infestans*, accessions collected from a range of geographically different areas were tested for their resistance under experimental field conditions. Furthermore, during our study, severe infections of bittersweet plants occurred in some accessions planted at Radboud University Botanical and Experimental Garden that allowed us to monitor the survival of *P. infestans* on *S. dulcamara* over the winter period.

Material and Methods

Monitoring of late blight on *S. dulcamara*

Five sites were selected for monitoring spots, four of natural occurring *S. dulcamara* in its habitat (Figure 1 A; site 1, 2, 3, 4; Figure 2) and one in an artificial setting where a collection was maintained as perennials (Figure 1 A; site 5). Monitoring started on the 1st of May and finished on the 30th of September 2006 and 2007. Every two weeks, these localities were monitored for symptoms of late blight with emphasis on regions where the highest concentration of Dutch organic potato cultivation is present, potentially causing high disease pressure on bittersweet (Figure 1 A; site 1, 2 ,3; Figure 2).

In these cases, monitored plants grew approximately 100 meters from the nearest potato fields. At site 4 (Figure 1 A), we expected a lower disease pressure on natural populations due to dry conditions and wind from the North Sea.

At site 5 (Figure 1 A), low disease pressure could be caused by a greater distance to nearest potato fields.

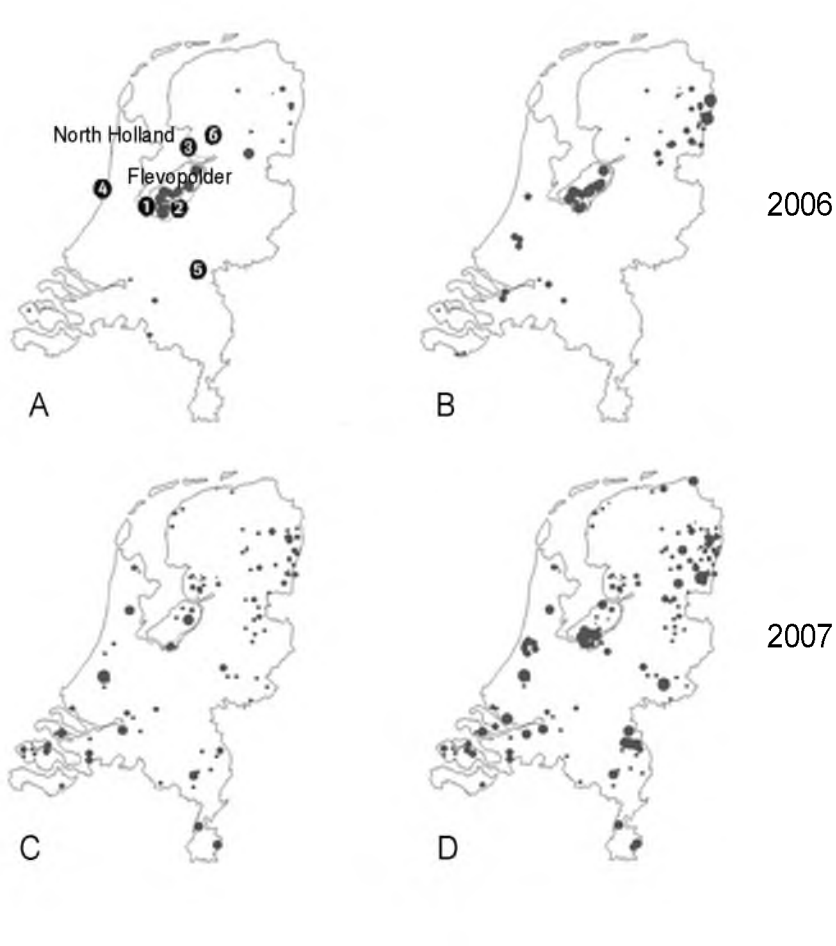

Figure 1. Map of The Netherlands with spots indicating late blight attacks reported on potato. The size of the dot indicates the severity of each outbreak. Figure A shows where the late blight was reported on 20.08.2006. Figure B shows where the late blight was reported on 05.09.2006. Figure C shows late blight appearance on 25.06.2007. Figure D shows late blight reported on 01.08.2007. Sites of *S. dulcamara* observed for natural infections during 2006 and 2007 season are marked on A. Site 1: Vicinity of Almerehout (bank of Gooimeer). Site 2: Zeewolde (bank of Veluwemeer) Site 3: South from Nagele (bank of Zwarte meer). Site 4: Castricum aan Zee (dune area). Site 5: Radboud University Botanical and Experimental Garden. Site 6: Experimental field where *S. dulcamara* accessions were artificially inoculated with *P. infestans*. The main concentration of organic potato production is located in the provinces of Flevoland and North Holland.

During the observation period, leaves that potentially showed late blight symptoms were collected and incubated for one week in a climate chamber (18°C; 16hrs day / 8hrs night) to promote disease development. Daily reports of weather conditions and late blight detections in The Netherlands during the investigated period were obtained from Dacom Plant Service (The Netherlands) (Figure 1).

Natural variation for resistance to late blight in accessions of *S. dulcamara*

Seeds of 56 *S. dulcamara* accessions from the Solanaceae collection of the Radboud University Botanical and Experimental Garden and seeds collected in 2005 and 2006 from *S. dulcamara* plants growing in their natural habitats mainly in The Netherlands, but also in Great Britain, Germany, Italy, Poland, Norway, New Zealand, Spain and USA were used (Table 1). Accession 94001 (Table 1; Figure 2), obtained in the course of another study (Golas et al. submitted) was used as a susceptible control. Additionally, cuttings were collected from a monitored plant growing in direct vicinity of potato fields (code: 206065; Figure 1 A; site 3). All accession numbers were abbreviated e.g.: accession number 944750001 is abbreviated to 94001 or A64750009 to A6009 and their passport data can be found at http://www.ru.nl/bgard. All plant material was cultivated in the greenhouse at the Radboud University Botanical and Experimental Garden under long day conditions (16hrs day / 8hrs night). Supplementary light was given by using high pressure sodium lamps (SON-T 600 W). Plants were grown in 1 liter pots filled with a standard soil mixture (Lentse Potgrond no.4). Regularly, the plants were fertilized with 2g/l with Kristallon Blauw (Yara Benelux B.V. Vlaardingen).

Approximately eight weeks old plants, one plant per accession, were chosen randomly for vegetative propagation. From the selected plants, cuttings with one node bud were taken, placed in wet soil and covered with plastic foil to increase humidity for a period of four days. A minimum of nine rooted cuttings were obtained per individual by the second half of May 2006 and 2007 respectively. On 22nd of June 2006 and 2007 *S. dulcamara* plants were planted on an experimental field near Marknesse (The Netherlands) (Figure 1 A; site 6), as a part of a much larger experiment, where potato breeding material was tested for late blight resistance. The experiment was designed in three randomized blocks, each block containing a plot of three plants. In the last week of July, spray inoculation was carried out with a suspension of *P. infestans* (complex A2 isolate IPO 82001, race 1,2,3,4,5,6,7,10,11 (Flier et al. 2003b)). Observations of disease development started three weeks after inoculation and lasted until the last week of September.

Table 1. Accessions of *S. dulcamara* used to screen the level of resistance under field conditions. The first and sixth column show an abbreviated accession code. The second and seventh columns indicate country of origin of the accession. The third and eight column indicate the habitat of the accession during sampling. The fourth and fifth and the ninth and tenth columns show a mean score of the resistance of the accession (n.t. – not tested). Resistance was scored on a scale from 3-9 where 3 is the most susceptible and9 the most resistant response. Values are overall means of composite scores in each of both seasons.

Accession code	Country of origin	Habitat	Resistance score 2006	Resistance score 2007	Accession code	Country of origin	Habitat	Resistance score 2006	Resistance score 2007
94001	Great Britain	unknown	3	3	A5247	The Netherlands	aquatic	7.33	7.22
206065	The Netherlands	aquatic	8.25	n.t.	A5249	The Netherlands	aquatic	7.47	7.39
A5102	The Netherlands	unknown	7.97	n.t.	A5250	The Netherlands	aquatic	6.14	6.78
A5174	The Netherlands	dunes	n.t.	8	A5252	The Netherlands	dunes	5.33	6.42
A5176	The Netherlands	unknown	7.25	8.22	A5254	The Netherlands	dunes	n.t.	8.75
A5177	The Netherlands	dunes	n.t.	7.61	A6008	Norway	unknown	n.t.	8.17
A5178	The Netherlands	dunes	n.t.	7	A6009	Norway	unknown	n.t.	7.22
A5179	The Netherlands	dunes	n.t.	6.33	A6015	Germany	unknown	n.t.	7.83
A5180	Germany	unknown	5.79	n.t.	A6016	Poland	unknown	n.t.	8.5
A5181	Italy	unknown	5.75	6.33	A6024	New Zealand	unknown	n.t.	7.44
A5183	Italy	unknown	7.19	7.67	A6030	Poland	unknown	n.t.	7.55
A5184	Poland	unknown	8.72	8.17	A6031	Spain	unknown	n.t.	5.78
A5187	The Netherlands	unknown	7.36	7.89	A6032	Spain	unknown	n.t.	7.72
A5188	The Netherlands	aquatic	n.t.	8.25	A6033	Spain	unknown	n.t.	7.61
A5189	The Netherlands	aquatic	n.t.	7.94	A6049	The Netherlands	dunes	n.t.	6.78
A5195	Italy	unknown	n.t.	8.65	A6050	The Netherlands	dunes	n.t.	7.89
A5200	The Netherlands	unknown	7.03	n.t.	A6051	The Netherlands	aquatic	n.t.	8.75
A5204	The Netherlands	unknown	7.67	8.09	A6052	The Netherlands	aquatic	n.t.	8.28
A5205	The Netherlands	unknown	7.61	7.67	A6053	The Netherlands	aquatic	n.t.	9
A5206	The Netherlands	unknown	n.t.	7.72	A6055	The Netherlands	dunes	n.t.	7.28
A5207	The Netherlands	aquatic	n.t.	7.45	A6058	The Netherlands	dunes	n.t.	6.61
A5234	The Netherlands	aquatic	n.t.	8.69	A6059	The Netherlands	dunes	n.t.	7.72
A5235	The Netherlands	unknown	7.89	n.t.	A6060	The Netherlands	dunes	n.t.	6.72
A5236	The Netherlands	unknown	7.64	8.53	A6061	The Netherlands	dunes	n.t.	8.31
A5238	The Netherlands	unknown	n.t.	7.5	A6062	The Netherlands	dunes	n.t.	7.44
A5241	The Netherlands	unknown	n.t.	7.72	A6063	The Netherlands	dunes	n.t.	8.75
A5242	The Netherlands	unknown	7	8.72	A6064	The Netherlands	aquatic	n.t.	8.94
A5243	The Netherlands	dry	7.47	8.39	A6071	USA	aquatic	n.t.	7
A5245	The Netherlands	dry	7.86	8.06					

Four types of observations were made on a scale of 3 to 9. The amount of healthy tissue, severity of leaf drop, sporulation intensity and a score for total impression for overall resistance was given. Mean scores over these four values of observations were used as an indication of the resistance level to potato late blight.

As a cut off criterion between resistance and susceptibility of a given genotype value 7 was used. Plants with a mean score below 7 were considered as susceptible and plants with the score 7 or higher as resistant.

Overwintering potential of *P. infestans* on *S. dulcamara* plants

To maintain the collection of *S. dulcamara*, five plants of each of the 56 accessions (Table 1) were planted in the Radboud University Botanical and Experimental Garden in May 2006, and maintained throughout the year 2007. To confirm the presence of *P. infestans* on bittersweet accessions naturally infected by late blight in 2006 and 2007 (Table 2), binocular examinations for the presence of sporangia were carried out. Additionally, detached leaf assays were performed as described by Vleeshouwers et al. (1999) to confirm that isolates collected from diseased *S. dulcamara* plants were pathogenic on cultivated potato. Sporangial suspensions were obtained by rinsing leaves of infected *S. dulcamara* plants in water. Trays containing detached leaves of the susceptible cultivar Bintje were put into soaked with water florist foam, inoculated and kept in closed plastic boxes for a period of one week in a climate chamber set at 18°C (16 hrs day / 8hrs night). Inoculation was carried out by applying two 10□l drops of the suspension on the abaxial side of the leaves. Pathogenicity of isolates was estimated after 7 days.

Table 2. Accessions of *S. dulcamara* infected by *P. infestans* in Radboud University Botanical and Experimental Garden. The first column an abbreviated accession code. The second column is an average score of the field resistance in 2006, where 3 is the most susceptible and 9 the most resistant response, (n.t. – not tested). The third column describes symptoms of natural late blight infection in 2006. The fourth column is a mean score of the field resistance in 2007. The fifth column describes symptoms of natural late infection in 2007.

Accession code	Field resistance 2006	Degree of natural infection in 2006	Field resistance 2007	Degree of natural infection in 2007
A5250	6,14	lesions no sporulation / regrowth	6,78	not infected
A5252	5.33	sporulation / regrowth	6.42	sporulation / regrowth
A5179	n.t.	sporulation / regrowth	6,33	lesions no sporulation
94001	3	sporulation / no regrowth	3	sporulation / no regrowth

To study whether *P. infestans* is able of over-wintering on bittersweet, accessions naturally infected during 2006 (Table 2) were used. In the first week of March 2007 selected plants were covered with a plastic tent to increase humidity and promote development of potentially over-wintering *P. infestans* on *S. dulcamara*. Plants inside the tent were frequently sprayed with water. After the start of the vegetation period in spring, leaves and stems of bittersweet plants were checked daily for late blight symptoms.

Results

Monitoring of late blight on *S. dulcamara*

Visual examinations were carried out in regions where *S. dulcamara* is growing in high density, close to commercially grown potato crops (Figure 1 A, Figure 2) and in dunes on the shores of the North Sea, near Castricum aan Zee (Figure 1 A). Observations were carried out even when weather conditions were not favorable for the pathogen. In The Netherlands a hot and dry period with little risk for late blight outbreak occurred in May, June, July and second half of September of 2006. In August, the weather shifted to mild and rainy, conditions more favorable for late blight development. Disease pressure started to increase and on the 20th of August (Figure 1 A) 38 outbreaks of late blight infections were reported that finally increased to 93 on the 5th of September (Figure 1 B) and later started to decrease. Throughout the entire period, *S. dulcamara* growing in its natural environment remained free from the disease, and approximately 100 collected leaf samples carrying lesions resembling those of *P. infestans* upon further incubation did not confirm the presence of *P. infestans*.

During the last week of August 2006, four out of 56 *S. dulcamara* accessions previously planted in Radboud University Botanical and Experimental Garden were found to be naturally infected by late blight (Table 2). The first infections appeared at the time when late blight was well established on cultivated potato and in some regions of The Netherlands the aerial concentration of spores was estimated as high. This situation was four weeks after a period of optimal weather conditions for late blight infection had started, and three weeks after the first reports of late blight epidemics in commercial farming. When disease pressure lowered, bittersweet plants started to recover throughout the second part of September and October. Some berries, although formed, did not ripen before the end of growing season. Initial disease symptoms on bittersweet plants were found on green and healthy leaves with various severities. Lesions without sporulation, clearly sporulating lesions and collapsed plants were observed (Table 2). Presence of *P. infestans* on these plants was confirmed visually by using an optical binocular.

To further confirm the presence of the pathogen a detached leaf assay was performed by applying a suspension of zoospores rinsed from sporulating leaves of bittersweet on the leaves of susceptible cultivar Bintje. In all cases, after 7 days, clear and typical sporulation was observed. Throughout the entire 2007 growing season, visual examinations were continued. However, in May, the first half of June and in September the disease pressure was relatively low due to warm and dry weather conditions. Optimal weather conditions for late blight development prevailed in the second half of June and in July (Figure 1 C; D).

During this season, late blight pressure on both cultivated potato, and hence, on wild solanaceous species was significantly higher than in 2006 with 131 reported late blight outbreaks already on the 25th of June (Figure 1 C). The number of outbreaks reached a maximum number of 212 on the 1st of August (Figure 1 D). Notwithstanding the early start of the epidemics, the higher number of late blight outbreaks and the overall longer period of favorable weather conditions than those of 2006, again, leaves of bittersweet with symptoms resembling late blight, upon further incubation, did not sporulate under laboratory conditions. Thus, infections were not detected on *S. dulcamara* in its natural habitat.

In 2007, at Radboud University Experimental and Botanical Garden three out of four accessions infected in 2006 were attacked again. Only the most resistant accession from the previously infected group showed no disease symptoms (A5250; Table 2). The first infections of *S. dulcamara* in appeared on the 5th of July 2007, and more infections appeared on already infected plants until approximately the 1st of August. After that day, we did not find late blight on *S. dulcamara* anymore, and except for plants of accession 94001, all plants recovered. Some small berries were formed on recovered shoots, but in comparison to healthy plants, there were less.

Despite the higher disease pressure in 2007 compared to 2006 (Figure 1), the severity of infections in the Radboud University Botanical and Experimental Garden was relatively weaker. Similarly to the pervious season, disease outbreaks started three weeks after epidemics in commercial potato farming had been recorded.

Natural variation for resistance to late blight in accessions of *S. dulcamara*

To study the level of resistance that is present in natural bittersweet populations, a set of 21 and 52 accessions was screened for resistance to late blight under field conditions in 2006 and 2007 respectively (Table 1). In 2006, 16 accessions had been collected in The Netherlands and 5 in other European countries. A monitored plant coded 206065 (Figure 1; Figure 2) collected near Emmeloord (The Netherlands) was included into the field experiment and showed no signs of late blight (resistance score 8.25), whereas the susceptible clone 94001 scored a value of 3.00, which was the lowest score of all tested genotypes in 2006 (Table 1; Figure 2).

In fact the plants of this genotype were killed by the pathogen. A majority of the accessions tested in 2006 showed a high level of resistance against late blight, but susceptible reactions were present as well. In total, 16 accessions showed a high level of field resistance with scores of 7 or higher, whereas the remaining 5 accessions scored less than 7. From the set of accessions collected outside The Netherlands, the Polish accession A5184 with the resistance score 8.72 and the Italian accession A5183 with the resistance score 7.19 were the most resistant genotypes tested in 2006.

The remaining accessions from Germany (A5180A) and Italy (A5181) were found to be susceptible to the pathogen with the resistance scores 5.79 and 5.75 respectively.

In general, the Dutch accessions were very resistant to the pathogen. Clone 206065 was the most resistant genotype present in the Dutch part of the collection with a resistance score of 8.25. Other resistant accessions were representing various regions and habitats throughout The Netherlands. The most susceptible Dutch accession A5252 with the resistance score 5.33 had been collected in the dune area of the western part of The Netherlands near Castricum aan Zee. A second rather susceptible accession A5250 (resistance score 6.14) was collected in Schardam (province of North Holland). Except the susceptible control 94001, all diseased plants recovered by re-sprouting when disease pressure lowered in the second half of September. Differences in the susceptibility to the late blight among accessions could not be directly ascribed to geographical origin or the habitat in which a particular accession had been collected (Table 1). The group of resistant genotypes represented various collection sites such as the dunes along the North Sea, the provinces of North Holland, Flevoland and eastern parts of The Netherlands. However, the most susceptible accession was collected in a dune area where potato cultivation is not present and disease pressure is lower, whereas the most resistant plant (code: 206065) was growing in direct vicinity of a potato field in an area of intense potato production.

In 2007, 52 accessions were tested, 37 from The Netherlands and 15 from other European countries, New Zealand and USA (Table 1). As in 2006, accession 94001 was included as a susceptible control and again in 2007 this plant showed the lowest level of resistance with an average disease score of 3.00. Similarly to the 2006 field trial, the majority of the accessions exhibited a high level of resistance to *P. infestans*.

Forty-three accessions scored a resistance level at 7.00 or higher. A low level of resistance was observed in 9 accessions with a disease score below 7.00. The 12 accessions collected outside The Netherlands had disease scores of 7.00 or higher and three scored lower than 7.00. The most resistant accessions of foreign origin were from Italy (A5195) and Poland (A6016) with resistance scores 8.5 and 8.65 respectively. An Italian accession A5181 again was classified as susceptible to late blight. A second susceptible accession A6031 was from Spain with a disease score of 5.78.

Out of 37 Dutch accessions, 31 were resistant to late blight. Within that group, six accessions had the highest resistance level observed during the two years of experimentation. The six most susceptible accessions were originated from the Dutch collection. The lowest level of resistance was again observed among genotypes collected in dune areas where five out of these six most susceptible accessions had been collected. The sixth accession A5250 was from Schardam (North Holland) and had been scored as susceptible in 2007 as well.
To evaluate the possible differences in mean resistance values as a result of seasonal variation, 16 accessions that had been screened in 2006 were also tested in 2007 (Table 1). Results showed a high and positive linear correlation [r=0,92] indicating that field trials were reproducible over both seasons.

Overwintering potential of *P. infestans* on *S. dulcamara* plants

Three out of four individuals (except 94001 that did not survive 2006 infection) naturally infected by late blight in 2006 at Radboud University Botanical and Experimental Garden were used to test whether *P. infestans* can survive winter on bittersweet plants (Table 2). Sprouts gradually appearing between March and end of April of 2007 were every day visually evaluated for disease symptoms. In total three individuals produced 31 shoots, some of which originated from the roots as they were emerging through the soil. Despite continuous observations and conditions favoring pathogen development for prolonged time every shoot produced was found healthy.

Discussion

In the present study all *S. dulcamara* plants growing in its natural environment along water channels, in vicinity of organically grown potatoes or in dunes were found to be resistant to late blight. Despite collection of many leaves with symptoms resembling late blight, we could not confirm the presence of the pathogen. In the study of Cook et al. (2002) repeated natural infections were found on a single plant growing less than 100 meters from the infected experimental plot of potato. In our case, two stretches of *S. dulcamara* (Figure 1; site 1, 2) each containing at least 50 plants were growing at a similar distance from organically cultivated potato, but representatives of these proved to be highly resistant. Until now, there are only few reports describing blighted bittersweet plants in nature (de Bary 1876; Flier et al. 2003). The rareness of such reports and the lack of positive results in this study indicate that susceptibility in this species is rather unique, and that the impact of bittersweet on overall late blight epidemiology must be minimal. Similar conclusions were drawn by Flier et al. (2003), who stated that the low frequency of natural infections observed, can not significantly contribute to overall late blight epidemiology.

It is highly probable that very susceptible plants are eliminated at an early stage of development (or even at seedling stage) as we have noticed for susceptible *S. dulcamara* (accession 94001). We noticed that natural infections in the Radboud University Botanical and Experimental Garden occurred only on representatives of accessions that had been scored in the field as belonging to the susceptible range of the spectrum. Apparently, most *S. dulcamara* plants are capable of surviving the epidemics that occur in potato.

A similar situation was described for Mexican species of *S. demissum* and *S. verrucosum* by Rivera-Peña (1990a). However, infection severely impairs fitness and reproduction of plants that are relatively susceptible, lowering their share in the population. It is plausible to attribute this process merely to the frequently observed late blight lesions on young stems that cause these to collapse, thus obstructing the formation of inflorescence and berries. Although nodal regrowth later in the season occurs, this retardation prevents susceptible plants from forming the berries that would be comparable in number to more resistant plants. Hence, their contribution to the offspring generation is anticipated to be significantly lower.

The infections observed at the Radboud University Botanical and Experimental Garden confirmed that *S. dulcamara*, like the cultivated potato is a host of late blight. Infections on bittersweet appeared when late blight was well established on cultivated potato and the disease pressure was high. This situation was observed both during the 2006 and the 2007 seasons when infections by *P. infestans* were appearing on *S. dulcamara* approximately three weeks after the first infections reported in commercial potato farming. As soon as the disease pressure lowered, moderately susceptible plants recovered. Similarly to our observations, Rivera-Peña (1990b) in Mexico observed late blight infections earlier on susceptible cultivars and then on wild *Solanum* species. Grünwald et al. (2001) also observed that epidemics on wild *Solanum* rarely occur before the end of August or beginning of September. This was the time when late blight had already well established in commercial cultivation. In North America, Deahl et al. (2004; 2006) and Olanya et al. (2005) also noticed blighted wild *Solanum* species in the vicinity of already heavily blighted potato fields.

We observed a remarkable dissimilarity in severity of attacks in 2006 and 2007 at Radboud University Botanical and Experimental Garden. Despite higher disease pressure in The Netherlands in 2007 (Figure 1) accessions suffered less than the same accessions during 2006. This difference could be caused by seasonal changes in *P. infestans* population structure and virulence pattern. The prevailing population of *P. infestans* in 2007 might have had a different virulence spectrum than in 2006 or could have been less adapted to bittersweet. It is also possible that *P. infestans* when migrating from its original host, potato, might not have been well adapted to a different host like *S. dulcamara*. This situation was described by Garry et al. (2005) who observed that the oomycete was more aggressive on its original host *S. tuberosum* than on *S. caripense* Dunal.

Results from the field experiments point in the direction that the regions were *S. dulcamara* accessions were collected might have some influence on the observed frequency of susceptible and resistant individuals in the bittersweet population. We have observed more susceptible genotypes in the part of the population that was collected in the dune area, especially in 2007.

By contrast, the most resistant plant (code: 206065) was collected in direct vicinity to a potato production area further supporting this statement. However, since resistant and susceptible reactions were also present among accessions from dunes and potato producing areas respectively, the resistance level of *S. dulcamara* plants is not strictly region specific.

Based on our observations, *S. dulcamara* cannot support living *P. infestans* throughout the winter nor be responsible for starting the infections early in the season. Despite thorough observations of plants that had been naturally infected in the previous season, we did not see any signs of late blight on fresh bittersweet sprouts emerging in the spring under conditions that favor *P. infestans*.

Despite sanitary precautions taken by farmers, late blight threatens potato cultivation each season and therefore, the role of weedy Solanaceae in initiating epidemics has been questioned frequently. We concluded that *S. dulcamara* infections in nature are at most scarce and hence not highly relevant in late blight epidemiology. The species seems also not as a reservoir for overwintering the inoculum from the previous cropping season and does not seem to initiate epidemics that occur during the potato vegetation period. However, it is clear that bittersweet is a good host, can suffer like cultivated potato from the attacks of the pathogen.

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Figure 2. On the left a stretch of *S. dulcamara* plants growing between *Angelica sylvestris* L., *Phragmites australis* (Cav.) Trin. ex Steud and *Urtica dioica* L. along the dam of the Gooimeer (Figure 1 A; site 1). On the right a susceptible control (94001; in front) and a resistant genotype (206065; in the back) in the experimental field trial at Marknesse (Figure 1 A; site 6).

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Identification of resistance gene *Rpi-dlc1* to *Phytophthora infestans* in European accessions of *Solanum dulcamara*

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Abstract

Initial screening of 14 *S. dulcamara* accessions enabled the identification of individuals resistant and susceptible to *Phytophthora infestans*. Crosses between contrasting genotypes resulted in three F_2-BC_1 populations segregating for resistance to late blight in a laboratory assay and under field conditions. Genetic profiling of one of these populations using 128 AFLP primer combinations generated three markers linked to the resistant phenotype. Blast analysis of the sequenced markers resulted in a plausible gene position on the distal end of the long arm of chromosome 9 that could be confirmed by CAPS markers. Thus, we describe a first resistant gene, named *Rpi-dlc1*, from *S. dulcamara*, a *Solanum* species native to Europe. Additionally, one population was tested for broadness of resistance responses using a set of seven additional *P. infestans* isolates, varying in virulence. This indicated the possible presence of additional *Rpi* genes.

Introduction

Of all diseases, late blight caused by *Phytophthora infestans* (Mont.) de Bary poses the biggest threat to cultivated potato worldwide. Today, commercial potato crops are mainly protected by frequent application of fungicides (Fry 2007). However, the costs for fungicides and their application are considerable and their negative impact on the environment is now recognized (Haverkort et al. 2008). In many parts of the world outside Mexico, which is considered the center of the greatest genetic diversity of the pathogen, a strong increase in genetic variability has been observed since the mid 1980's (Goodwin and Drenth 1997).

The apparent capacity of the pathogen to develop resistance to modern fungicides (Goodwin et al. 1996; Grünwald et al. 2001) necessitates constant vigilance by farmers. Therefore breeders have been extremely interested in creating resistant cultivars from the beginning of the twentieth century onwards. The first breeding activities mainly focused on dominant resistance genes, as the complete resistance they conferred was easy to follow and promised a fast and effective way to protect crops against late blight. Single dominant resistance genes were initially identified in the Mexican species *S. demissum* and rather easily crossed into cultivated potato. By the 1970's, 11 such genes had been introgressed (Gebhardt and Valkonen 2001). However, their durability proved to be a problem, since virulent races of the pathogen appeared quickly, in a few cropping seasons, after market introduction (Wastie 1991) or even earlier, during the breeding process (Muller 1951). Nevertheless, by the mid-twentieth century, late blight was kept at a tolerable level, mainly by agricultural practices including moderately resistant cultivars (Fry and Goodwin 1997).

In the last two decades, progress in molecular biology allowed insight into the chromosomal positions of most of the genes introduced from *S. demissum* into *S. tuberosum* (Leonards-Schippers et al. 1992; Li et al. 1998; Huang et al. 2004, 2005; El-Kharbotly et al. 1994, 1996; Bradshaw et al. 2006) and two genes have now been cloned and characterized: *R1* and *R3a* (Ballvora et al. 2002; Huang et al. 2005). Currently, apart from *S. demissum*, other wild American species of the genus *Solanum* are considered as possible sources of resistance, but introgression of these genes into cultivars often requires interspecific bridge crosses. In the Mexican species *S. bulbocastanum* three genes were identified; the allelic *RB* (Helgeson et al. 1998; Naess et al. 2000; Song et al. 2003) and *Rpi-blb1* (Van der Vossen et al. 2003) on chromosome 8, *Rpi-blb2* on chromosome 6 (Van der Vossen et al. 2005) and *Rpi-blb3* on chromosome 4 (Park et al. 2005). In the wild species *S. pinnatisectum* Dunal a dominant gene *Rpi1* was mapped by Kuhl et al. (2001) to chromosome 7. On chromosome 9, genes from *S. mochiquense* Ochoa named *Rpi-mcq1*, *S. phureja* Juz. and Buk. named *Rpi-phu1* and *S. venturii* Hawkes and Hjert. named: *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3*, were identified (Smilde et al. 2005; Sliwka et al. 2006; Foster et al. 2009; Pel et al. 2009). Two *S. berthaultii* genes *Rpi-ber1* and *Rpi-ber2* were mapped on chromosome 10 (Park et al. 2009). Also *S. microdontum* Bitter, *S. paucissectum* Ochoa and *S. stoloniferum* Schltdl. are considered as resistance sources (Sandbrink et al. 2000; Villamon et al. 2005; Tan et al. 2008; Wang et al. 2008). All of these species are tuber bearing *Solanum* species of section *Petota*. Up to now, only one resistant gene has been described in the non-tuber bearing representatives of the genus *Solanum*: the South American species *S. caripense* Dunal carries a major *R*-gene that has been mapped on chromosome 9 (Trognitz and Trognitz 2004; Nakitandwe et al. 2007). In Europe, in contrast to the new world, only few native solanaceous species are present. Among them are *S. nigrum*, an annual hexaploid weed, and the perennial diploid *S. dulcamara*, also known as bittersweet or climbing nightshade. Bittersweet is an easy to recognize, out-crossing species with a low level of genetic variation, which occupies a range of habitats (Golas et al. submitted). In The Netherlands, it can often be found in the vicinity of commercial potato fields. *Solanum dulcamara* has been known for a long time as a host for *P. infestans* under natural conditions (de Bary 1876), but it hardly suffers from late blight attacks, even under conditions highly favorable for disease outbreaks that cause severe crop losses of organically produced potatoes (Golas et al. submitted).

The goal of this study was to unravel the genetic factors that cause the observed high level of resistance of *S. dulcamara* against *P. infestans* present in individuals from natural populations. The screening of diploid *S. dulcamara* accessions usually revealed a strong resistance, but occasionally susceptibility.

The availability of both resistant and susceptible variants made it possible to start a genetic study on the nature of resistance to late blight in this European species. This paper describes the identification, field assessment, and mapping of a first resistance gene named *Rpi-dlc1,* in three accessions of *S. dulcamara*.

Material and methods

Plant material

Fourteen *S. dulcamara* accessions (Table 1), each represented by five individuals, were obtained from the collection of the Botanical and Experimental Garden of Radboud University Nijmegen (The Netherlands). Accessions, together with developed segregating mapping populations were cultivated at the Botanical and Experimental Garden, Nijmegen, The Netherlands. Passport data concerning the accessions used can be found at http://www.ru.nl/bgard. Seeds were sprayed with GA_3 (Duchefa, The Netherlands) and germinated at 30^{o}C on moist soil.

Table 1. Accessions of *S. dulcamara* used to screen the level of resistance under field conditions and in detached leaf assays in summer 2004. The first column is an abbreviated accession code. The second column indicates the country of origin of the accession. The third column indicates the number of resistant/susceptible individuals within each accession under laboratory and field conditions.

		Resistance screening	
Accession code	**Country of origin**	**R**	**S**
85003	Germany	4	1
88034	Austria	5	0
88057	France	5	0
91008	Switzerland	5	0
91046	Italy	5	0
91081	France	4	1
92023	Belgium	5	0
92109	France	5	0
92195	Poland	5	0
93233	The Netherlands	5	0
94001	Great Britain	3	2
A4081	Unknown	5	0
A4088	Unknown	5	0
A4148	Poland	5	0

After two weeks, seedlings were transplanted to 1 liter pots filled with a standard soil mixture (Lentse Potgrond no. 4). Plants were grown in the glasshouse under long day conditions (16 hrs day/8 hrs night) with supplementary light of high pressure sodium lamps (SON-T 600 W) and were regularly fertilized with 2g/liter of Kristallon Blauw (Yara Benelux B.V. Vlaardingen). During winter, plants were kept in a cold glasshouse.

Crosses

All crosses that led to F_1 and F_2-BC_1 populations and the increased population 05-346 were performed under greenhouse conditions in the winter of 2004, the summer of 2005 and the summer of 2006. Just before opening, flowers were emasculated and hand pollinated the next day. In all cases, the susceptible parent was used as a pollen donor. Six weeks after pollination, seeds were extracted from mature berries and stored in paper bags at 4°C.

In the present study, three crosses were used to investigate the *Rpi-dlc1* resistance gene. Cross 05-188: [(92023-4_{Rp} x 94001-2_{Sp}) x 94001-2_{Sp}] was constructed using a resistant parent, identified within the 92023-4 (Rp) accession collected in Belgium at Bel Herbeumont, and a susceptible parent 94001-2 (Sp), collected in Great Britain at St. Aldhelm's Head. To obtain population 05-203: [(88057-2_{Rp} x 91081-4_{Sp}) x 94001-5 $_{Sp}$] a resistant parent 88057-2 (Rp) collected at Duinkerken (France) was firstly crossed with a susceptible plant 91081-4 (Sp) collected in France at Fort-Mahon-Plage and than with another susceptible parent 94001-5 (Sp), collected in Great Britain at St. Aldhelm's Head. Cross 05-346: [(A4148-3_{Rp} x 94001-2_{Sp}) x 94001-2_{Sp}] contains a Polish resistant genotype collected at 5033—N and 01927—E crossed twice with the susceptible parent 94001-2 (Sp).

Phytophthora infestans isolates

Phytophthora infestans isolates: 90128, Ipo82001 collected in The Netherlands and Belgium respectively (provided by V. Vleeshouwers Wageningen University, The Netherlands) and isolate Ipo655 collected in The Netherlands (Table 2) were used in detached leaf assays (DLAs).

Table 2. Virulence spectrum of *P. infestans* isolates used in detached leaf assays and field experiments. The virulences of the isolates used were determined using the *S. demissum* derived set of differentials *R1-R11*.

Isolate	Virulence profile
T30-4	0
88133	0
MP806	1,3,4
MP722	1,2,3,4
88069	1,2,3,4,6,7
US618	1,2,3,4,6,10
90128	1,2,3,4,6,7,10,11
89148.09	1,2,3,4,6,7,10,11
Ipo82001	1,2,3,4,6,7,10,11
Ipo655	1,2,3,4,5,6,7,10,11

For characterization of the broadness of the resistance spectrum, an additional set of seven isolates was kindly provided by F. Govers (Wageningen University, The Netherlands) (Table 2). The virulence of each isolate was determined by performing detached leaf assays with the *S. demissum* differentials set *R1-R11* obtained from Wageningen University (Flier et al. 2003).

Resistance screening

For the initial field trial, *S. dulcamara* plants approximately eight weeks old: (five plants per accession) were vegetatively propagated in April 2004. Cuttings with one node bud were obtained, placed in moist soil and covered with plastic foil to increase humidity for a period of 4 days. A minimum of three rooted cuttings was obtained per plant and potted in the second half of May 2004. On the 22nd of June 2004, three plant plots were planted on an experimental field near Marknesse (The Netherlands), as a part of much larger trial, where potato breeding material was tested for late blight resistance. On the 28th of July, spray inoculation was carried out with a suspension of *P. infestans* (complex A2 isolate Ipo82001, race 1,2,3,4,5,6,7,10 and 11 (Flier et al. 2003)). Subsequently, segregating populations and individuals of an extended population coded 05-346 were screened under field conditions in the years 2005 to 2008. Plants were propagated and planted as described above, but nine rooted cuttings were produced per individual, instead of three (plots were planted in three randomized blocks with each plot containing three plants). Planting, inoculation and resistance assessment were done essentially as in the previous year. Observations of disease development were carried out from mid of August till end of September, as in 2004. Four types of observations were made: the estimated amount of green and healthy tissue present on the plants, the estimated severity of yellowed and dropped leaves, the sporulation intensity and the total impression of plant fitness. Each of these parameters were given a score on a scale from 3 to 8, where 3 indicates the most susceptible/unhealthy plant and 8 the most resistant/healthy plant. A value for a field resistance of a given individual was calculated as the average score for all observations made during a testing season. The score of 3 was the lowest score measured for a susceptible phenotype, and a score of 8 indicated immunity to late blight.

Detached leaf assays (DLas) were performed as described by Vleeshouwers et al. (1999). Fresh sporangia were produced in a weekly cycle on detached leaves of the susceptible potato cultivar Bintje. Trays with leaves put into water soaked florist foam were kept in closed plastic boxes for a period of one week in a climate chamber set at 18^{o}C (16 hours day / 8 hours night). A fresh suspension of zoospores was produced by rinsing around five infected leaflets in approximately 200 ml tap water with a few drops of raw potato tuber sap added to it.

After incubation of the sporangial suspension at 4°C to induce zoospore release, inoculation was carried out by applying two 10 µl drops of suspension on the abaxial side of the leaves. Infection severity was evaluated 7 days after inoculation. Leaves showing clear sporulaton were classified as susceptible whereas leaves showing a necrotic response at the site of the inoculation or a lesion without sporulation were classified as resistant.

DNA isolation and genetic analysis

Total genomic DNA was isolated from young leaves using the Wizard genomic DNA purification kit (Promega), according to the protocol supplied by the manufacturer. A pestle was used to grind approximately 40mg of fresh plant material in liquid nitrogen to a fine powder. After extraction the concentration of DNA was measured using a spectrophotometer (Pharmacia Biotech: GeneQuant II) and the quality of the DNA was checked by electrophoresis in a 1% agarose gel (Eurogentec, Belgium) stained with EtBr.

Amplified fragment length polymorphism (AFLP®) analysis was performed according to Vos et al. (1995). Initial digestion of total genomic DNA was done using *Eco*RI and *Mse*I restriction enzymes (Fermentas, Germany). Pre-amplification and selective PCR was performed using thermocycler GeneAmp9600 (Perkin Elmer, USA). Visualization of selective PCR products was done by labeling EcoRI primers with radioactive gamma-^{33}p (MP Biomedicals, USA). Labeled selective PCR products were separated on a 5% polyacrylamide gel (Duchefa, The Netherlands), dried on paper and visualized by exposure to X-ray film (Kodak BIOMAX MR) for 48 hours. Gels were scored manually.

The bulk segregant analyses (BSA) approach was used (Michelmore et al. 1991) in order to obtain AFLP fragments co-segregating with resistance in mapping populations. Two bulks were constructed by combining DNA of five, either resistant or susceptible individuals. DNA samples in each bulk were mixed after the pre-amplification step in equal concentrations. Bulks and parental DNA samples were analyzed using 128 MseI/EcoRI AFLP primer combinations. Primer combinations that yielded an AFLP product only in the resistant parent and in the resistant bulk but not in the susceptible parent and in the susceptible bulk were identified and applied to all the individuals of a segregating population. AFLP fragments linked to the resistant phenotype were excised from the gel and subsequently, DNA was eluted and re-amplified under the same conditions as for the pre-amplification. PCR products were cloned into pGEM-T Easy (Promega, USA) and sequenced using the CEQ™ DTCS Quick Start Kit (Beckman Coulter 8000™).To develop markers in the region of *Rpi-dlc1* sequence information from GABI (http://www.gabipd.org/database/maps.shtml) and SGN (http://sgn.cornell.edu/cview/map.pl?map_id=9&show_offsets=1&show_ruler=1) databases were used for primer design.

Markers used for mapping the *Rpi-dlc1* gene were also applied to the diploid potato mapping population SHxRH (Van Os et al. 2006; provided by H. van Eck Wageningen University, The Netherlands) containing 101 offspring. Polymorphic markers were searched by digestion of PCR generated fragments using 12 tetra-cutter restriction enzymes (*Alu*I, *Hpa*II, *Rsa*I, *Dpn*II, *Mse*I, *Bsu*RI, *Hha*I, *Nla*III, *Dde*I, *Hinf*I, *Hpy*CH4IV, *Taq*I). Genetic distances in cM were calculated based on the number of recombinants. Publicly available potato and tomato genetic maps from the GABI and SGN were included for comparison of marker positions and synteny.

Results

Populations segregating for the resistance

An initial screening of the 14 *S. dulcamara* accessions, challenged under field conditions in the year 2004 with Ipo82001 and in DLA with isolates Ipo82001 and 90128, revealed a considerable level of variation in the response to *P. infestans*. Within the tested set of accessions, both resistance and susceptibility to late blight among individuals was identified (Table 1). Under field conditions, the most resistant individuals remained free of symptoms. On less resistant accessions, infections appeared on young stems and mature leaves that later dropped off. On two highly susceptible genotypes from accession 94001, intensive sporulation was observed and those plants were killed. In DLA, localized necrosis at the site of inoculation and lesions without noticeable sporulation were observed on resistant genotypes. On moderately resistant and susceptible individuals a clear sporulation 7 days after inoculation was observed. In order to develop segregating populations, 39 crosses were made between resistant and susceptible genotypes of *S. dulcamara*. F_1 populations were tested in DLA and one population, 05-40 (92023-4_{Rp} x 94001-2_{Sp}) containing 49 offspring clones was tested in the field in 2005. In DLA, F_1 populations generally did not segregate in a reproducible manner and no clear segregation was observed for population 05-40 (data not shown). Also, the field test conducted in 2005 gave no clear segregation in population 05-40, but rather a broad phenotypic range that was skewed towards susceptibility (Figure 1). Further, the susceptible parent had a low score of 3 and the resistant parent the highest score of 8. Offspring plants showed phenotypes that were all intermediate in comparison to the parents.

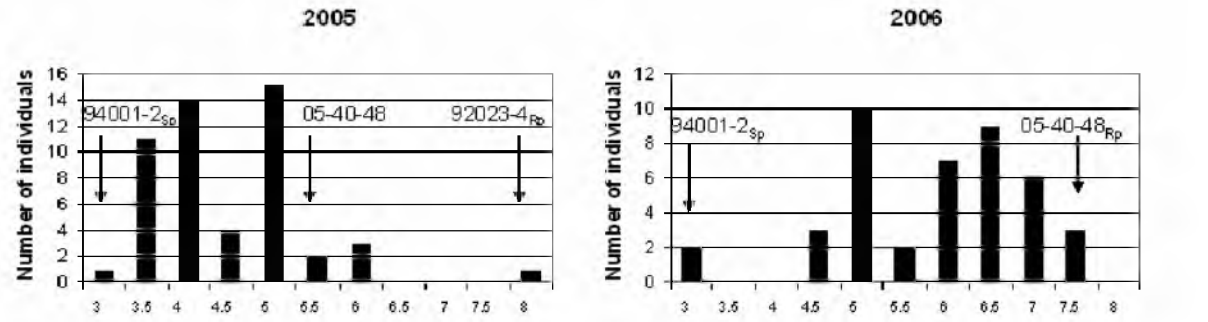

Figure 1. Distributions of the resistant phenotypes in F_1 cross 05-40 containing 49 plants (left side) and in BC_1 cross 05-188 containing 40 plants (right side) under field conditions. On the X axis is indicated mean disease severity where 3 is most susceptible and 8 is most resistant. On the Y axis the number of individuals classified to each phenotypic group is given.

From each F_1 population, the resistant individual(s) based either on DLA or field data were selected and crossed again to one of the available susceptible parents. Forty four BC_1 populations obtained in this way were tested in DLA. Population 05-188, was obtained after backcrossing the resistant individual no. 48 from population 05-40 to the susceptible parent 94001-2. This population, containing 40 individuals, was selected for a field test carried out in the growing season 2006, to track the changes in response to the late blight between the F_1 and BC_1 generation. In general the resistance level of the 05-188 offspring plants was scored significantly higher than that of population 05-40. In the previous season, population 05-40 showed a weak binominal distribution for field resistance, whereas population 05-188 displayed rather clear segregation, close to a 1:1 (χ^2=3; P=0.083) of susceptible individuals having the resistance score of 5.5 or lower and resistant individuals scoring 5.5 or higher (Figure 1).

In another pair of BC_1 populations, coded 05-203 and 05-346 containing 45 and 52 individuals respectively, clear and reproducible segregation in DLA was observed (population 05-203: χ^2=0.35; P=0.55; population 05-346: χ^2=1.92; P=0.16; DLA data not shown). Both populations were segregating roughly according to a 1:1 ratio after inoculation with isolates 90128 or Ipo82001 in replicated DLas.

Molecular analysis of segregating populations

AFLP analyses were conducted on population 05-203 and this ultimately yielded three co-segregating fragments: two at 5cM, and one fragment at 2cM distance from a gene that putatively was designated *Rpi-dlc1*. Primers were designed based on the sequence of these AFLP markers and tested on a pre-amplification mix to confirm whether the proper fragments were isolated.

Two out of three primer pairs reproduced the original segregation pattern of the AFLP markers, thereby confirming correct cloning. These two confirmed markers (eACTmCAC with primer pair 5'-3' – catggcttcccgtactgaat and 5'-3' – tcacctttccaggcaaaaac and eACTmCGC 5'-3' – cgcacaatttgtgcatcgcg and 5'-3' - cactgtagtagcatatttgg) were tested on genomic DNA of population 05-203 and both amplified a product of the expected size in all individuals, but the amount of PCR product was considerably lower in the susceptible parent, susceptible offspring and recombinants. As a next step toward the mapping of *Rpi-dlc1*, the sequences of the markers eACTmCAC and eACTmCGC were blasted against plant genomic sequences available in public databases. The highest sequence homology of the AFLP fragment amplified by eACTmCAC (Accession number: FJ769334) was with the *S-adenosyl-L-homocysteine hydrolase* gene from *S. tuberosum*, found in the GABI database, indicating two possible positions for *Rpi-dlc1*: one on potato chromosome 9 between markers GP41 and CT220 and the other on chromosome 12 between markers GP122 and GP264. To verify both putative genomic positions of *Rpi-dlc1*, four primer pairs were developed based on available sequences of these markers. Pairs yielding clear PCR products were used to search for polymorphisms between the parental genotypes of population 05-203. Therefore PCR products were digested with a set of 12 restriction enzymes. As a first result, marker GP41, after digestion with *Rsa*I, revealed a polymorphism between the parents of population 05-203. Subsequently, GP41/*Rsa*I was tested on the entire population 05-203 and was indeed found to co-segregate with the phenotypic scores. Positions on chromosome 12 did not yield a polymorphic band. Subsequently from available databases, a set of 28 markers, previously mapped in the region of GP41 in potato or tomato, were selected and primers were developed on the basis of their available sequences. In total, 16 primer pairs yielded amplification products in *S. dulcamara* and 4 of them (S2g3/*Alu*I; TG591A-L/*Bsu*RI; CT220est/*Nla*III; S1d11/allele specific) were polymorphic and co-segregated with resistance in population 05-203 (Table 3).

Table 3. Markers that were found to co-segregate with the *Rpi-dlc1* gene on chromosome 9 in three independent segregating populations of *S. dulcamara*. N.p. – no polymorphism, n.d. – not determined, a.s. – allele specific.

				Population/rest. enzyme			
Marker	**Primer (5'-3')**	**Tm(oC)**	**Fragment size (bp)**	**05-188**	**05-203**	**05-346**	**source**
S2g3	GGGTGTGAATACTTTTACTC CAATGTAAAAACTCAAGCCC	48	800	*Alu*I	*Alu*I	n.p.	GABI
TG591A-L	ACAATCGCTAGCGGTATACC GAAACTTTATAGGCACGTCC	54	500	*Bsu*RI	*Bsu*RI	n.p.	SGN
GP101	GGCATTTCTATGGTATCAGAG GCTTAACATGCAAAGGTTAAA	52	750	n.d.	n.p.	*Alu*I	GABI
GP41	CAGGAGATCCATCTCTCAAG CTGCAGTAAAGTGCATTCGG	51	1200	n.d.	*Rsa*I	*Alu*I	GABI
CT220est	AGAGTAGTGTGATTGAGACG GATGATCATCGCAGTAGAGG	48	900	n.d.	*Nla*III	n.p.	SGN
T0521	CAGTCGGCGCAGTTTCAAAA GCATTACCTAGATCAATGCC	59	1300	n.d.	n.p.	*Dde*I	SGN
S1d11	GTAACTCTTCCTATTGTACTC CCAATAGTCATTGTAAGGCGC	58	300	n.d.	a.s.	a.s.	GABI
S1d5-a	CGCCTCTTTCTCTGAATTTC GATCTGGGATGGTCCATTC	57	550	n.d.	n.p.	a.s.	GABI

The other two segregating populations, 05-188 and 05-346, were also tested for co-segregation at the *Rpi-dlc1* locus with the set of markers used in population 05-203 (Table 3). In both populations, segregation of the identified polymorphisms was consistent with phenotypic data. In population 05-346, originally consisting of 52 offspring clones, recombinants were identified between *Rpi-dlc1* and a set of five available polymorphic markers. This population was subsequently enlarged to 302 individuals to generate a more detailed map of the *Rpi-dlc1* locus. In total, 23 crossing-over events were identified between markers GP101 and the T0521 marker (7.6cM). T0521 is positioned more distal from the centromere than marker GP101. However, due to the loss of seven of these recombinant genotypes, only 16 could eventually be phenotyped in the field and tested with the available remaining three polymorphic markers. To be able to compare genetic distances obtained for GP101 and T0521, genetic distances between S1d5-a, GP41 and S1d11 were calculated for 210 individuals/16 recombinants (Figure 2 B). In the enlarged population 05-346 each marker appeared to be separated by at least one crossing over.

This allowed narrowing down the genetic region containing *Rpi-dlc1* by comparing the presence/absence of a marker with resistance/susceptibility of the recombinant. Between markers S1d11 and T0521 one resistant recombinant was identified containing all markers except T0521, excluding the possible presence of *Rpi-dlc1* near T0521. Recombinant containing T0521 and S1d11 could not be used due to the lack of reliable phenotypic results. Subsequently, three resistant recombinants were identified that contained GP101, S1d5-a and GP41 markers, but not S1d11.

Additionally a susceptible recombinant was identified that contained GP41, S1d11 and T0521, but not GP101 and S1d5-a, thus excluding the GP41 region. Finally two sets of recombinants; one containing only GP101 and second containing all other markers except GP101 were found to contain both resistant and susceptible genotypes, indicating that the *Rpi-dlc1* locus is localized somewhere between the GP101 and S1d5-1 markers (Figure 2 B). Population 05-346 was also used to compare the genomic region of the *Rpi-dlc1* locus of *S. dulcamara* to a corresponding region in the potato SHxRH mapping population The five markers used to map the *Rpi-dlc1* locus in population 05-346 were successfully mapped on the genetic map of potato SHxRH (Figure 2 A). All markers were mapped in the same order in *S. dulcamara* and SHxRH except S1d5-a, and GP41, where inversion between these markers was detected. Significant differences were also present in the calculated genetic distance between markers GP101 and S1d5-a (Figure 2 A).

Broadness of resistance

To test the spectrum of resistance governed by *Rpi-dlc1*, 20 resistant and 20 susceptible genotypes from population 05-346 were tested in DLAs with a set of eight *P. infestans* isolates varying in virulence profile (Table 2). Isolates 90128 and Ipo82001 were used for reference. The resistant parent 142-12$_{Rp}$ of 05-346 was found to be resistant to all isolates whereas the susceptible parent 94001-2$_{Sp}$ appeared to be susceptible to all isolates. The *Rpi-dlc1* gene in resistant individuals of population 05-346 was found functional to isolates 90128, Ipo82001 and additionally to T30-4. Phenotypes scored upon infection with 88069, 88133 and 89148.09 were skewed towards susceptibility (resistance/susceptibility 9/31; 4/36; 8/32 respectively). More resistant individuals were found when isolates MP722 and Ipo655 were used (13/27 and 16/26 resistant/susceptible respectively). A 1:1 segregation was detected with isolates US618 (19/21) and MP806 (21:19), although segregation in resistant and susceptible individuals was not according to the assumed presence or absence of *Rpi-dlc1*. Individual 346-41 from this population was resistant to all tested isolates, and 346-57 was susceptible only to isolate US618 (Table 4).

Table 4. Results from the detached leaf assay of resistant parent (142-12Rp), susceptible parent (94001-2Sp) and 20 resistant and 20 susceptible individuals from population 05-346 inoculated with 10 *P. infestans* isolates. R-resistant phenotype, S-susceptible phenotype.

	Phytophthora infestans									
Genotype	**90128**	**T30-4**	**Ipo82001**	**88069**	**MP722**	**88133**	**89148.09**	**US618**	**MP806**	**Ipo655**
142-12Rp	R	R	R	R	R	R	R	R	R	R
94001-2Sp	S	S	S	S	S	S	S	S	S	S
346-1	R	R	S	S	S	S	S	S	S	S
346-2	R	R	S	S	R	S	S	S	S	S
346-3	R	R	S	S	R	S	S	R	S	S
346-4	R	R	S	S	R	S	S	S	S	S
346-5	S	S	S	S	R	S	S	R	S	S
346-6	R	R	S	R	R	S	S	R	S	S
346-7	S	S	S	S	S	S	S	R	R	S
346-8	R	R	S	S	S	S	S	S	S	S
346-10	S	S	S	S	R	S	S	S	S	S
346-11	R	R	S	R	R	S	S	S	R	S
346-12	S	S	S	R	R	S	S	R	R	S
346-21	S	S	S	S	S	S	S	R	R	S
346-22	R	R	S	S	R	S	S	R	S	S
346-23	R	R	S	R	R	S	S	R	R	S
346-25	S	S	S	S	S	S	S	R	R	S
346-26	R	R	S	S	S	S	S	R	R	S
346-27	R	R	S	S	S	S	R	S	R	R
346-28	S	S	S	S	S	S	S	S	S	R
346-29	S	S	S	S	S	S	S	S	S	R
346-31	R	R	S	S	S	S	S	S	S	R
346-32	S	S	S	S	S	S	S	R	R	R
346-34	R	R	S	R	S	S	S	R	R	R
346-36	S	S	S	S	S	S	R	R	R	R
346-37	S	S	S	S	S	S	S	R	R	R
346-38	S	S	S	S	S	R	S	S	S	S
346-39	S	S	S	S	S	S	S	S	R	S
346-40	S	S	S	S	S	S	S	S	R	S
346-41	R	R	R	R	R	R	R	R	R	R
346-42	S	S	S	S	S	S	S	S	R	R
346-43	S	S	S	S	S	S	S	R	R	R
346-46	R	R	S	S	S	S	R	S	R	S
346-48	S	S	S	S	S	S	R	R	R	S
346-49	R	R	S	S	S	S	S	S	S	S
346-50	R	R	S	S	S	S	S	S	S	R
346-52	S	S	S	S	S	S	S	S	S	S
346-53	S	S	S	S	R	R	R	R	S	R
346-57	R	R	R	R	R	R	R	S	R	R
346-58	R	R	R	R	S	S	S	S	S	R
346-59	S	S	S	S	S	S	S	S	R	R
346-60	R	R	S	R	S	S	S	R	S	S

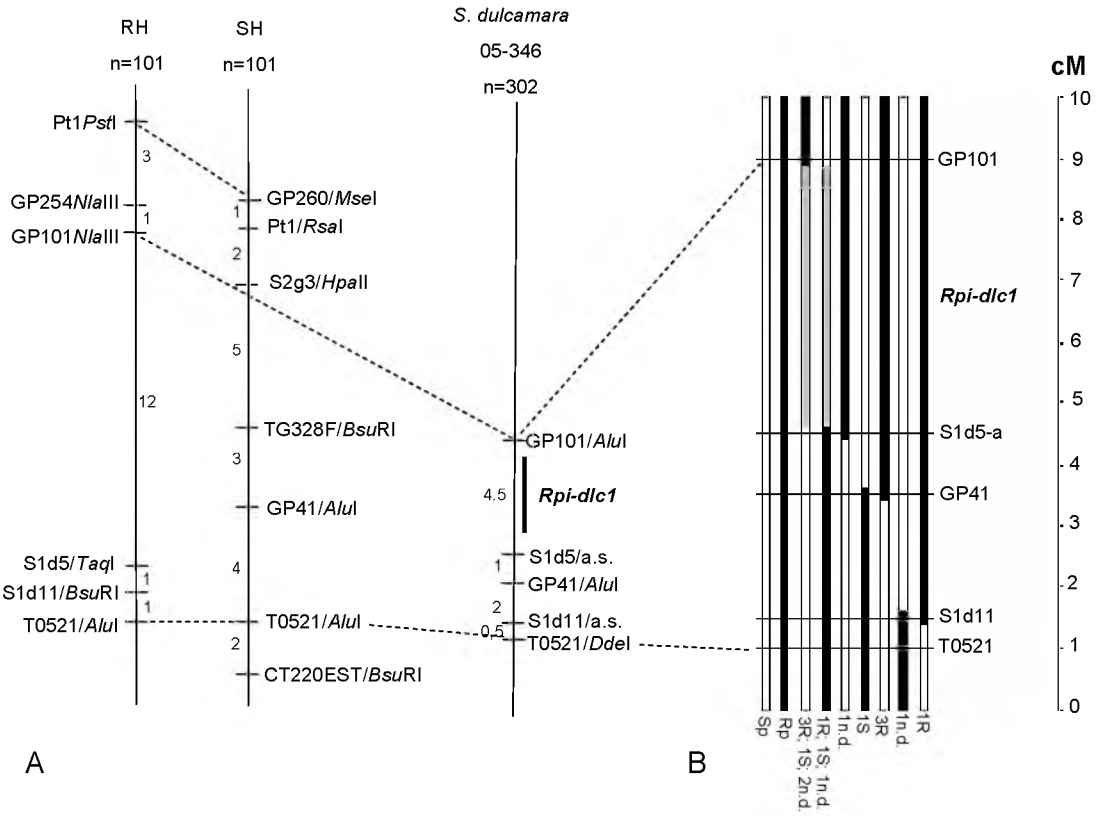

Figure 2. (A) Maps of chromosome 9 of potato (RHxSH) and *S. dulcamara* near the *Rpi-dlc1* locus. Numbers indicate genetic distances in cM between markers. (B) Schematic representation of the genomic region in the population 05-346 containing *Rpi-dlc1*. Black bars represent resistant, white bars susceptible marker profiles and grey bars indicate the genomic region where *Rpi-dlc1* was mapped. At the bottom end of the bars results from the field experiment are presented. Numbers indicate number of offspring with clear phenotype, R is resistant, S susceptible and n.d. not determined phenotype. Rp and Sp are the resistant and susceptible parents respectively. Genetic distances between markers GP101 and T0521 were calculated for 302 individuals. Among these, 23 recombinants were found. Genetic distances for the remaining three polymorphic markers S1d5-a, GP41 and S1d11 were calculated using 210 individuals and 16 recombinants that were tested in the field. On the right a cM scale.

Discussion

The goal of this study was to identify the genetic factors responsible for the elevated level of resistance as observed in *S. dulcamara* accessions against *P. infestans* (Golas et al. submitted). In this paper we have described the identification, field assessment, and mapping of a first resistant gene of *S. dulcamara* against late blight that is designated *Rpi-dlc1*. Here, we follow the system of Van der Vossen et al. (2003) for naming newly identified resistance genes against late blight. This system suits for easy connection of the resistance gene with the species in which it was originally identified.

Renewed interest in *R*-gene based resistance against late blight resulted in the identification of numerous wild *Solanum* species as valuable sources of resistance, other than the previously exploited *S. demissum*. However, for some *Solanum* species, high levels of resistance or non host resistance create a difficulty in the sense that susceptible genotypes suitable for genetic studies are not available. Problems of that nature also apply to the European species *S. dulcamara* and *S. nigrum* (Cooke et al. 2002; Flier et al. 2003; Lebecka 2008). In the case of bittersweet, susceptibility to late blight was reported earlier, but it is relatively rare, and often a susceptible reaction in the detached leaf assay experiments does not necessarily indicate full susceptibility under field conditions. In our initial experiments, we used isolates of *P. infestans* that had a rather broad spectrum of virulence and were considered as aggressive (Flier et al. 2003). The resistance assays on various accessions of *S. dulcamara* were done both in the field and under laboratory conditions and resulted in the identification of susceptible *S. dulcamara* genotypes having a stable, reproducible, highly susceptible phenotype. We used a similar approach in parallel to identify susceptible individuals in accessions of *Solanum* species of section *Solanum* (*S. nigrum*, *S. sarrachoides*, *S. scabrum* and *S. villosum*), but were unable to identify reliable susceptible plants (Golas, unpublished results) and we failed to set up populations in these species that segregated for resistance to late blight.

Currently, there is limited overall knowledge about *S. dulcamara*, and this species was never considered as a possible source of resistance against late blight. Also, to our knowledge, the genetic basis of resistance to *P. infestans* in *S. dulcamara* was not known. In several solanaceous crop plants, especially potato and tomato, resistance genes to different pathogens have been identified and placed on the genetic maps. The majority of them group together in clusters of genes called “resistance hot spots” (Gebhardt and Valkonen 2001). *Rpi-dlc1* was found to map within such a cluster on the long arm of chromosome 9, where resistance genes to various pathogens have been mapped (Brommonschenkel and Tanksley 1997; Tommiska et al. 1998; Rouppe van der Voort et al. 2000; Chunwongse et al. 2002; Trognitz and Trognitz 2004; Smilde et al. 2005). *Rpi-dlc1* appeared to map in the proximity of another gene, *Rpi-mcq1* from the tuber bearing species *S. mochiquense* (Smilde et al. 2005). However, the RFLP marker TG328, found to be linked to *Rpi-mcq1* did not amplify in any of *S. dulcamara* resistant parents used to develop the segregating populations. This indicates that the region of *Rpi-mcq1* is not present in *S. dulcamara* and therefore might suggest that *Rpi-mcq1* and *Rpi-dlc1* are different loci. However, their eventual relationship requires additional fine mapping. Comparison of the marker order in this region on *S. dulcamara* chromosome 9 with *S. tuberosum* showed that *S. dulcamara* shares a basic level of genetic synteny with these species. However, markers that were mapped on the genetic map did show some inversions in *S. dulcamara*.

Moreover, most of the markers localized on the genetic map of potato did not differ significantly from the genetic distances observed in *S. dulcamara* using the same markers. However, only about half of the tested markers could be amplified in the *S. dulcamara* genome. This situation indicates that there is some divergence caused by lack of basic sequence similarity between potato, tomato and bittersweet in that region, which is logic in the light of the taxonomic position of *S. dulcamara* relative to *S. tuberosum* and *S. lycopersicum* (Weese and Bohs 2007).

Although the phenotypes of population 05-188 obtained from the field showed a binominal distribution, plants from the resistant group of the segregating population were not immune to late blight. Based on that, we concluded that this gene behaves as a QTL under field conditions and this gene has rather a slowing down effect on disease progress, than that it confers immunity. Possessing a QTL nature, *Rpi-dlc1* was difficult to track in DLA experiments, thus field data were necessary to obtain clear phenotypes. The tested broadness of resistance using one of the segregating population with additional isolates revealed the possibility of the presence of additional *Rpi* genes, different from *Rpi-dlc1*.

Rpi-dlc1 or at least allelic versions of the gene were identified in three independent populations derived from parents originating from geographically different regions of Europe (France, Belgium and Poland). Experiments that have been carried out to study the genetic variation of European *S. dulcamara* revealed high molecular similarity (above 90%) between accessions, even among those geographically separated (Golas et al. submitted). Finding the same gene (or its allelic versions) present in three populations originating from distant genotypes supports the high similarity existing among the bittersweet populations described earlier. This result might indicate that this gene is widely distributed and that it helps to protect *S. dulcamara* from late blight attacks during the growing season. Because this gene does not provide immunity to the disease, it is likely that other, even more resistant individuals identified by us in parallel field assays (data not shown) possess additional resistance genes that are different from the one described here. *Rpi-dlc1* is the first resistance gene or strong QTL identified in a *Solanum* species native to the Eurasian continent. Thus, our result shows that not only the Solanaceae of Americas are a source of resistant genes to late blight.

The current literature shows that there is no indication that *S. dulcamara* coevolved with *P. infestans* until around 1840's when this oomycete arrived in Europe. The devastation of cultivated potato could have been accompanied by attacks of late blight on native *Solanum* species such as *S. dulcamara* and *S. nigrum*. De Bary (1876) already noticed attacks on shaded leaves of *S. dulcamara*, but he did not mention massive disease on individuals in nature.

We can think of several hypotheses that might explain the observation of *S. dulcamara* being a species capable of defending itself against a completely new pathogen through functional *R*-genes. Firstly, it is possible that *R*-genes were recruited from a general reservoir of functional *R*-genes that must have been present at the time of arrival and subsequent epidemics from 1845's onwards. Likely these genes were present in the population at low frequency, thus massive attacks must have occurred in the natural populations of *S. dulcamara*, leaving a population merely resistant. This then could explain the high genetic similarity and lack of clear structure in bittersweet populations as we observed (Golas et al. submitted). However, to our knowledge there are no reports of massive attacks of *S. dulcamara* plants in 1845 or later. This was probably due to lack of knowledge of a true cause of the disease. Only from the 1870's people became aware of *P. infestans* being the cause of late blight.

Secondly, *R*-genes like *Rpi-dlc1* could operate against different, yet unknown pathogens, but with a pleiotropic effect on late blight. An example of such a situation is the *Mi*-gene in tomato that is functional against the nematode *Meloidogyne incognita* as well as aphids and white flies (Goggin et al. 2001; Nombela et al. 2003).

A third, but less likely hypothesis, is that *P. infestans* was present in Europe long before 1845. Hence *S. dulcamara* and other European solanaceous weedy species could already have been evolutionary adapted and capable of resisting infection. Then it has to be hypothesized that recorded epidemics in potato were not results of a newly introduced pathogen, but rather an adaptation of *P. infestans* from a non to a highly pathogenic status with regard to the potato as a susceptible host. However, *P. infestans* sequences from herbarium material collected during the 1850's epidemics in Ireland link the European strain of *P. infestans* directly to the South American population (May and Ristaino 2004; Gomez-Alpizar 2007).

In conclusion, in this paper we have described the first R locus that confers resistance to *P. infestans* in *S. dulcamara*, an European *Solanum* weed. Another *Solanum* species widely distributed in Europe is *S. nigrum*, in which resistance to *P. infestans* was recently described by Lebecka (2009). Also in *S. nigrum*, as in *S. dulcamara*, the resistance seems to be conferred by a single locus, although in *S. nigrum* the genetic position is still unknown. Our results, however, indicate that in *S. dulcamara* other Rpi genes are present, similarly to the tuber-bearing American *Solanum* species. Comparison of all these *Rpi* genes may in the future help to elucidate the evolutionary processes that shaped resistance in plants.

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Identification of resistance gene *Rpi-dlc2* to *Phytophthora infestans* in European accessions of *Solanum dulcamara*

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Abstract

Screening of 20 accessions of *Solanum dulcamara* with *Phytophthora infestans* isolate Ipo82001 in the laboratory by leaf assay revealed strong resistance present in an individual belonging to accession A54750069. A F_1 population obtained from a cross made between this plant and a susceptible genotype contained 63 individuals that segregated in 1:1 ratio both in detached leaf assays as in a field experiment. Presence of the formerly mapped *Rpi-dlc1* gene as a cause of the observed resistance was excluded. Subsequently, AFLP analyses using 196 primer combinations enabled identification of 5 markers linked to the resistance gene putatively named *Rpi-dlc2*. Blast analyses of the sequenced fragments showed highest homology to potato/tomato sequence on chromosome 4, 8 and 11; however, different approaches to map the gene on either of these chromosomes did not provide a definitive genetic location. Here we describe identification of the second resistant gene *Rpi-dlc2* for late blight resistance identified in *S. dulcamara*, a *Solanum* species native in Europe. Additionally, a cross containing *Rpi-dlc1* and *Rpi-dlc2* resistance genes from *S. dulcamara* was made and tested under field conditions. In general individuals containing both resistance genes showed to be significantly more resistant to *P. infestans* than those with either one or none.

Introduction

Solanum dulcamara L., commonly named bittersweet or climbing nightshade, is one of the about 1500 species of the cosmopolitan genus *Solanum* L. (D'Arcy 1991; Weese and Bohs 2007). It belongs to the section *Dulcamara* (Moench) Dumort. of subgenus *Potatoe*, that also includes the cultivated potato and its wild relatives (Child and Lester 2001). *Solanum dulcamara* is a diploid, perennial plant, one of few native Solanaceae present in Europe, although during the last few centuries it has been naturalized in many parts of the world. *Solanum dulcamara* is an easy to recognize, out-crossing species with a low level of genetic variation (Golas et al. submitted; 2009a). It occurs in diverse habitats, from shaded river banks and lake shores to dry and exposed dunes and plains. Additionally, bittersweet is often found in the vicinity of commercial potato fields where it could serve as a reservoir for pathogens harmful in potato production like *Ralstonia solanacearum* (Smith) Smith, a causal agent of bacterial wilt (Olsson 1976; Elphinstone et al. 1996; Janse 1996).

Solanum dulcamara has been known for a long time as a host for *Phytophthora infestans* (de Bary 1876). However, even favorable weather conditions for late blight only result in sporadic infections (Flier et al. 2003a; Cooke et al. 2002; Dandurand et al. 2006).

Understanding the nature of the apparent resistance in the European population of *S. dulcamara* could help to understand how Solanaceae plants organize their defense under intense pressure of the late blight pathogen. Recently, a first resistance gene named *Rpi-dlc1* against *P. infestans* was identified and mapped on a chromosome of *S. dulcamara* equivalent to chromosome 9 of potato/tomato (Golas et al. submitted; 2009b).

In the present study, we describe the identification of a second resistance gene from *S. dulcamara*. This resistance gene, putatively designated as *Rpi-dlc2*, in contrast to *Rpi-dlc1*, confers a strong monogenic resistance to *P. infestans*. Additionally, a population containing both *Rpi-dlc1* and *Rpi-dlc2* was evaluated under field conditions, revealing the highest level of resistance in individuals containing two genes rather than those containing either one or none.

Material and methods

Plant material

Seeds of 15 *S. dulcamara* accessions, each represented by five individuals, were obtained from the collection of the Radboud University Botanical and Experimental Garden (The Netherlands). Additionally, four genotypes (each treated as an accession) collected as cuttings in the spring 2005 from plants growing in natural settings at the Thames in Great Britain were included. Individuals 2 and 5, each susceptible to late blight, from accession 944750001 were used as controls in resistance assays and as pollen donors in crosses (Table 1). Accessions and derived populations, were cultivated at the Radboud University Botanical and Experimental Garden. For germination, seeds were sown on moist soil, sprayed with GA_3 (Duchefa, The Netherlands) and placed in a 30°C chamber. To regenerate plants from collected cuttings, parts of stem containing node bud were placed in moist soil and covered with plastic foil to increase humidity for a period of four days. Then cover was removed and after additional ten days, seedlings and rooted cuttings were transplanted to one liter pots filled with a standard soil mixture (Lentse Potgrond no. 4). Plants were grown in the glasshouse under long day conditions (16 hrs day / 8 hrs night) with supplementary light of high pressure sodium lamps (SON-T 600 W), and were regularly fertilized with 2g/l of Kristallon Blauw (Yara Benelux B.V. Vlaardingen). During winter, plants were kept in a cold glasshouse.

Table 1. Accessions of *S. dulcamara* selected after the initial DLA screening for field experiment. The first column shows the most resistant individuals after DLA. The second column indicates the country of origin of the accession, n.d. not determined. The third column shows results obtained in DLA with disease severity ranging from the most susceptible V8, through V7, V6, V5, R6, R7 to R8, being the most resistant response. In the field, mean disease severity ranged from one, meaning susceptibility to nine, the most resistant phenotype.

Accession code	Country of origin	Resistance screening		Accession code	Country of origin	Resistance screening	
		DLA	Field			DLA	Field
A44750147-3	Poland	R8	9	A54750008-2	The Netherlands	V6	5.5
A54750069	Great Britain	R8	8.75	A54750067	Great Briatain	V6	5.5
A54750002-1	Italy	R7	8.75	924750194-2	n.d.	V6	5.25
A44750151-3	Poland	R7	8.5	974750113-4	n.d.	V6	5.25
A54750003-3	n.d.	R6	8.25	A54750005-2	The Netherlands	R7	5.25
A44750149-3	Poland	R6	8	A54750008-4	The Netherlands	R6	5.25
A44750151-4	Poland	R8	8	A44750090-3	n.d.	V6	5
A44750149-4	Poland	R7	7.5	A44750182-4	n.d.	V5	5
A44750147-1	Poland	R7	7.5	924750194-1	n.d.	V6	4.75
A54750007-3	The Netherlands	V5	7.25	A44750081-1	n.d.	R6	4.5
A44750081-3	n.d.	V5	7	A44750182-1	n.d.	R6	4.5
A54750003-1	n.d.	R6	7	A54750002-5	Italy	V5	4.25
A54750007-1	The Netherlands	R8	7	A54750005-1	The Netherlands	R6	4
A54750066	Great Britain	R6	6.75	A24750105-4	n.d.	R6	3.5
A54750068	Great Britain	R6	6.75	A44750090-4	n.d.	V5	3.25
A54750009-4	The Netherlands	R7	6.25	A24750105-3	n.d.	V5	2.5
974750113-5	n.d.	R6	6.25	944750001-2	Great Briatain	V8	1.75
A54750009-1	The Netherlands	V5	5.75	944750001-5	Great Briatain	V8	1.75

Crosses

To develop the F_1 segregating mapping population 05-150 [A54750069$_{Rp}$ x 944750001-2$_{Sp}$], resistant parent A54750069, collected in Great Britain at the banks of Thames was crossed with a susceptible plant 944750001-2 collected in Great Britain, at St. Aldhelm's Head.

To obtain the F_2-BC_1 population 07-410, an individual of 05-150 was crossed with another susceptible individual from accession 944750001: [05-150-13$_{Rp}$ x 944750001-5$_{Sp}$].

To develop population 07-407, containing individuals carrying *Rpi-dlc1* and *Rpi-dlc2*, the resistant individual no. 50 from population 05-346 (Golas et al. submitted; 2009b) was crossed with resistant individual no. 19 from population 05-150: [05-346-50$_{Rpi\text{-}dlc1}$ x 05-150-19$_{Rpi\text{-}dlc2}$] All crosses were performed under greenhouse conditions in summer 2005 for the F_1, and in summer 2007 for F_2-BC_1 and 07-407.

Just before opening, flowers were emasculated and hand pollinated the next day. Six weeks after pollination seeds were extracted from mature berries, air dried and stored in paper bags at 4°C.

Phytophthora infestans isolates

Phytophthora infestans isolate Ipo655-2A race 1,2,3,4,5,6,7,8,9,10,11 (Flier et al. 2003b) collected in The Netherlands was used in detached leaf assays (DLas). For testing the populations under field conditions complex A2 isolate Ipo82001 race 1,2,3,4,5,6,7,10,11 (Flier et al. 2003b) was used.

Resistance screening

Detached leaf assays (DLA)

DLAs were performed as described by Vleeshouwers et al. (1999). Fresh sporangia were produced in a weekly cycle on detached leaves of the susceptible potato cultivar Bintje. Trays with leaves put into water soaked florist foam were kept in closed plastic boxes for a period of one week in a climate chamber set at 18°C (16 hrs day / 8 hrs night). A fresh suspension of zoospores was produced by rinsing ca five leaflets in approximately 200 ml tap water with a few drops of raw potato tuber sap added to it. After incubation of the sporangial suspension at 4°C to induce zoospore release, inoculation was carried by applying two 10 µl drops of suspension on adaxial side of the leaves. Infection severity was evaluated seven days after inoculation. A plant was considered as susceptible when leaves showed clear sporulation (V8 or V7), less intense sporulation (V6) or sporulation was observed under binocular (V5). A plant was scored as resistant when no sporulation was seen on developed necrotic lesions (R6), lesions were limited (R7), or only at site of inoculation (R8).

Field trials

For each experiment, approximately eight weeks old plants were vegetatively propagated in April. A minimum of nine rooted cuttings were obtained per individual and potted in the second half of May.

Around the 22nd of June 2005, 2006, 2007 and 2008, three plant plots each containing three plants were planted on an experimental field near Marknesse (The Netherlands), as a part of much larger trial, where potato breeding material was tested for late blight resistance. In the end of July, spray inoculation was carried out with a suspension of *P. infestans* isolate Ipo82001. Observations of disease development were carried out from mid of August till end of September.

Four types of observations were made: the estimated amount of green and healthy tissue present on the plants, the estimated severity of yellowed and dropped leaves, the sporulation intensity and the total impression of plant fitness. Each of these parameters were given a score on a scale from 1 to 9, where 1 indicates the most susceptible/unhealthy plant and 9 the most resistant/healthy plant. A value for a field resistance of a given individual was calculated as the average score for all observations made during a testing season.

DNA isolation and genomic profiling

Total genomic DNA was isolated from young leaves using the Wizard genomic DNA purification kit (Promega, USA), according to the protocol supplied by the manufacturer. A pestle was used to grind approximately 40mg of fresh plant material in liquid nitrogen to a fine powder. The concentration of DNA was measured using a spectrophotometer (Pharmacia Biotech: GeneQuant II) and the quality of the DNA was checked by electrophoresis in a 1% agarose gel (Eurogentec, Belgium) stained with Ethidium bromide.

A set of four CAPS markers linked to *Rpi-dlc1* resistance gene was used to exclude the presence of *Rpi-dlc1* in population 05-150 (Golas et al. submitted; 2009b). The same set of markers (except TG591A-L) was used to screen population 07-407 for the presence of *Rpi-dlc1*. Amplified fragment length polymorphism (AFLP®) analysis was performed according to Vos et al. (1995). Initial digestion of total genomic DNA was done using *Eco*RI and *Mse*I restriction enzymes (Fermentas, Germany). Pre-amplification and selective PCR was performed using thermocycler GeneAmp9600 (Perkin Elmer, USA). Visualization of selective PCR products was done by labeling EcoRI primers with radioactive gamma-^{33}p (MP Biomedicals, USA). Labeled selective PCR products were separated on a 5% polyacrylamide gel (Duchefa, The Netherlands), dried on paper and visualized by exposure to X-ray film (Kodak BIOMAX MR) for 48 hours. Gels were scored manually. The bulk segregant analyses (BSA) approach was used (Michelmore et al. 1991) in order to obtain AFLP fragments co-segregating with resistance in mapping populations. Two bulks were constructed by combining DNA of five, either resistant or susceptible individuals. DNA samples in each bulk were mixed after pre-amplification step in equal amounts.

Bulks and parental DNA samples were analyzed using 196 (population 05-150) and 128 (population 07-410) MseI/EcoRI AFLP primer combinations.

Primer combinations that yielded an AFLP product only in the resistant parent and the resistant bulk, but not it in the susceptible parent and the susceptible bulk were identified and applied to all the individuals of segregating populations.

AFLP fragments linked to the resistant phenotype were excised from the gel and subsequently, DNA was eluted and re-amplified under the same conditions as for the pre-amplification. PCR products were cloned into pGEM-T Easy (Promega, USA) and sequenced using the CEQ™ DTCS Quick Start Kit (Beckman Coulter 8000™).

To map AFLP markers, potato mapping population SHxRH (Van Os et al. 2006; provided by H. van Eck, Wageningen University, The Netherlands) and a set of tomato introgression lines (provided by S. van Heusden, Wageningen University, The Netherlands) were used.

To search for polymorphisms linked to *Rpi-dlc2*, a set of 72 SSR markers (provided by H. van Eck, Wageningen University, The Netherlands) developed for *S. tuberosum* with known genetic position was tested on both S. *dulcamara* segregating populations.

For developing new sets of CAPS markers in an attempt to confirm chromosomal position of th *Rpi-dlc2*, sequence informations from GABI (http://www.gabipd.org/database/maps.shtml) and SGN (http://sgn.cornell.edu/cview/map.pl?map_id=9&show_offsets=1&show_ruler=1) databases were used for primer design. Additionally 05-150 and 07-410 populations were tested with a set of about 700 markers (about 60 markers per chromosome) designed based on sequences of markers available on GABI and SGN. Markers were randomly distributed throughout 12 chromosomes of potato/tomato. Polymorphic markers were searched by digestion of PCR generated fragments using 12 tetra-cutter restriction enzymes (*Alu*I, *Hpa*II, *Rsa*I, *Dpn*II, *Mse*I, *Bsu*RI, *Hha*I *Nla*III, *Dde*I, *Hinf*I, *Hpy*CH4IV, *Taq*I). Digestions were carried out for at least 2 hours and were checked on 1.5% agarose gels stained with EtBr. Alternatively, polymorphism was searched within PCR fragments shorter than 500bp and amplified with annealing temperature of 63^{0}C using LightScanner (Bioke, The Netherlands) for high-resolution DNA melting curve (Hi-Res Melting™).

Results

Population segregating for the resistance

15 *S. dulcamara* accessions each represented by five individuals, four accessions each represented by a single genotype and two susceptible plants used as control, derived from the same accession were challenged in detached leaf assay with *P. infestans* isolate Ipo655-2A (results not shown). This experiment revealed a wide range of responses to the inoculation, with susceptibility and resistance being often present within one accession.

Out of each accession, if available, two most resistant individuals were identified (Table 1). Highest level of resistance among all tested plants was found in four accessions; A44750147, A54750069, A44750151 and A54750007 scored as R8.

Five individuals from five accessions were scored as R7 and eleven individuals representing ten accessions were scored as R6 (Table 1).

Typical late blight symptoms were observed in 15 individuals belonging to 10 accessions. Susceptible control plants no. 2 and no. 5 from accession 944750001 were scored as V8. Six individuals from five accessions were scored as V6 and seven individuals from seven accessions were scored as V5 (Table1).

Based on results obtained from DLAs, all plants listed in table 1 were tested in the field experiment of 2005. As in DLAs, the field experiment revealed a range of responses to inoculation with *P. infestans*. Two individuals (no. 3 from accession A44750147 and the one individual of accession A54750069) again proved to be the most resistant individuals within the collection. Both plants had the highest resistance scores of 9 and 8.75 respectively, obtained during the experiment. Two other individuals, A44750151-4 and A54750007-1, although equally resistant in DLA, proved to be less resistant in the field experiment with scores 8 and 7, respectively (Table 1). Susceptible control plants from accession 944750001 were scored 1.75, the lowest score for all genotypes tested. Plants of that accession were killed by the pathogen. In general, plants scored as R6, R7, V5, V6 or V7 in DLAs were infected to a various degree during the field experiment by late blight, although plants scored as more resistant in DLAs showed a higher field resistance, although with some exceptions (Table 1).

As a next step in analyzing the genetic source of resistance, individual no. 3 from accession A44750147 and the single individual of accession A54750069 were crossed with a susceptible parent 944750001-2. Two populations obtained in that way were tested in DLA (not shown). However, only in case of a cross between A54750069 x 944750001-2, coded 05-150 and containing 63 individuals, reproducible 1:1 segregation with 29 resistant and 28 susceptible genotypes was observed. Further, this population was tested under field conditions in the season 2006 (Figure 1) and 2007. There also, it was clearly segregating into two groups. In the field, resistant parent and resistant offspring plants remained free of symptoms, whereas on the susceptible parent and susceptible genotypes intensive disease symptoms had developed. Phenotypic data obtained earlier in DLA were consistent with those scored in the field.

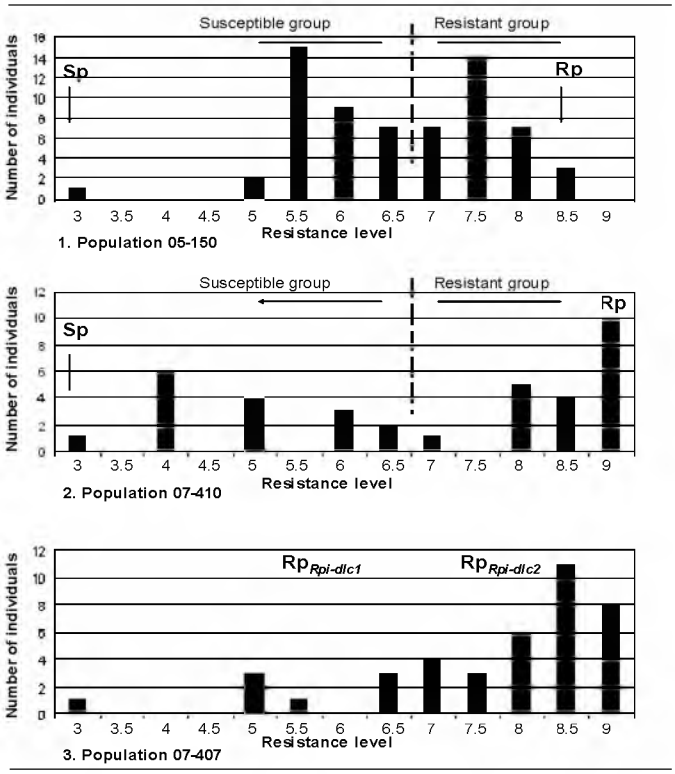

Figure 1. Distribution of the phenotypes in F_1 cross 05-150 containing 63 plants (upper graph) and in F_2-BC_1 cross 07-410 containing 34 plants (middle graph). Bottom graph shows distribution of phenotypes in cross 07-407 containing 37 plants. Sp, Rp, $Rp_{Rpi\text{-}dlc1}$, and $Rp_{Rpi\text{-}dlc2}$ above the respective bars indicate resistance level of parents of each population.

Similarly to population 05-150, F_2-BC_1 population 07-410 containing 34 individuals and population 07-407 containing 37 individuals were tested in DLAs and subsequently under field conditions (Figure 1). Population 07-410 was segregating into two clear groups of 19 resistant and 16 susceptible plants, and in case of 07-407 segregation was inclined towards the resistant spectrum (Figure 1).

Molecular analysis of segregating populations

As a first step towards mapping the gene in population 05-150, the presence of *Rpi-dlc1* was excluded by testing GP41, GP101, TG591A-L and T0521 markers linked with it (Golas et al. submitted; 2009b). Amplification of PCR products of expected size was obtained for all tested markers, however only PCR product of marker TG591A-L after digestion with restriction enzyme *Bsu*RI revealed a polymorphism in resistant parent. Subsequent analyses of that marker on the individuals of 05-150 population resulted in no co-segregation of the molecular marker with the phenotypic scores, thereby excluding the *Rpi-dlc1* as gene responsible for resistance.

Population 05-150 was then used for AFLP analyses that ultimately yielded three co-segregating fragments at 5cM (eAACmCTG, eACCmCGC, eAAGmCTG) and two co-segregating fragments at 16cM (eAACmCCA, eAACmCAC) distance from a gene that putatively was designated *Rpi-dlc2*. Two fragments, bigger than 100bp generated by eAACmCTG and eAACmCAC primer combinations were isolated and sequenced. In the meantime F_2-BC_1 population 07-410 containing 34 individuals was developed and tested with 128 AFLP primers. Four markers were identified at distances of 8cM (eACCmCGT), 11cM (eAATmCAT; eAACmCCA) and at 17 cM (eACGmCTT) from the gene. However, only the fragment amplified by eAATmCAT primer combination was isolated, sequenced and successfully converted into PCR based marker. Confirmation of the marker was done by performing PCR with primers designed based on the obtained sequences of isolated fragments and by checking for co-segregation on pre-amplification mix on genomic DNA of population 05-150 or 07-410.

All sequences of confirmed markers were blasted against plant genomic sequences available in public databases of GABI and SGN. The highest sequence homology of all three AFLP fragments was obtained with tomato sequences of chromosome 8 in the vicinity of marker GP40. High similarity of AFLP sequences was also obtained on potato chromosomes 4 near the *α-Glucosidase* (Agl) marker and on chromosome 11 near the *Nitrate reductase* (Nia) marker. All three chromosomal positions were checked by designing primers based on sequences of markers previously mapped in the vicinity of the indicated positions and by developing CAPS markers. In all cases CAPS markers either for 05-150 or 07-410 populations showed no co-segregation with the phenotypic data. Moreover the marker profile of CAPS developed in this way did not show linkage to AFLP markers linked to *Rpi-dlc2*.

As a next step an attempt was made to map AFLP markers on the genetic map using SHxRH potato mapping population and tomato insertion lines. To be able to obtain rough estimates of *Rpi-dlc2* one of *S. dulcamara* AFLP markers converted into PCR based markers should amplify in the reference population of SHxRH or in one of avialialbe tomato insertion lines. Identification of polymorphism within the generated PCR fragment or amplified region in tomao insertion lines would provide us with the genetic position of the marker. However apparent lack of homology of sequenced markers to both *S. tuberosum* and *S. lycopersicum* prevented us from obtaining good PCR fragments on which we could work further.

To search for polymorphisms linked to *Rpi-dlc2*, a set of 72 SSR markers was tested on both *S. dulcamara* segregating populations. Out of a set of these markers, 28 could be amplified in parents of both *S. dulcamara* segregating populations, and only three SSR markers were polymorphic, but also in that case no co-segregation was detected.

A further attempt was made using a set of about 700 markers designed on available sequences of potato/tomato genomes from SGN and GABI databases.

As a first step each marker was tested for amplification on parents of both populations using *S. tuberosum* or *S. lycopersicum* genomic DNA as control, to test whether a single fragment of equal size of that present in control plants could be amplified in *S. dulcamara*. These criteria were matched by 238 markers (34%) and they were used for re-amplification. Subsequently, PCR products were digested with a set of 12 restriction enzymes. Digested PCR products were checked for polymorphism on agarose gels. Out of 238 markers, 15 polymorphic ones were detected and subsequently were tested on the entire population of 05-150 or 07-410. None of the identified polymorphisms showed cosegregation with phenotypic data. Polymorphic markers were detected for all chromosomes with exception of chromosomes 5 and 10, where no polymorphic markers were identified.

Fourteen, out of 238 primer pairs amplifying in *S. dulcamara*, but not polymorphic after digestions with restriction enzymes were tested using high-resolution DNA melting curve. Parents of both segregating populations were tested and within this set, two additional polymorphisms were identified in resistant parent of population 05-150. Both markers were mapped to chromosome 11 in the vicinity of marker St1.2.1. However, when markers were tested on offspring of population 05-150 no co-segregation was obtained for the phenotypic data.

Phenotypic and molecular evaluation of population containing *Rpi-dlc1* and *Rpi-dlc2*

In total, 37 offspring plants of population 07-407 were tested in the field experiment of 2008 (Figure 2). Based on phenotypic results, these plants were divided into four groups. The first group contained 18 individuals immune to late blight with resistance score above eight. The second group of eight individuals was classified as resistant with resistance scores above seven to eight. The third slightly susceptible group contained eight plants scored above six to seven. The fourth, most susceptible group had three plants with resistance score up to six (Figure 2).

Table 2. Individuals of population 07-407 with corresponding resistance level and marker profiles. N.d. – not determined.

		Marker profiles:					
		Rpi-dlc1			***Rpi-dlc2***		
Individuals	**Resistance scores**	**T0521/*Dde*I**	**GP41/*Alu*I**	**GP101/*Alu*I**	**eACC/mCGT**	**eAAT/mCAT**	**eACC/mCAC**
1	6.5	1	1	1	0	0	0
2	8.5	0	n.d.	0	1	1	1
3	8.5	1	1	1	1	1	1
4	8.25	1	1	1	1	1	1
5	8.4	1	1	1	1	1	1
6	8.8	1	1	1	1	1	1
7	8.45	1	1	1	1	1	1
8	5.3	0	n.d.	0	0	n.d.	0
9	7.54	0	0	0	1	1	1
10	8.45	0	0	n.d.	1	1	1
11	7.81	1	1	1	0	0	0
12	5.2	0	0	0	0	0	0
13	9	0	0	0	1	1	1
14	5.1	0	0	0	0	0	0
15	6.9	0	0	0	0	n.d.	0
16	6.8	1	1	1	0	0	0
17	8.7	0	0	1	1	1	1
18	8.6	1	1	1	1	1	1
19	8.3	0	0	1	1	1	1
20	6.1	1	1	1	0	0	0
21	7.3	0	0	0	0	0	0
22	8.63	1	n.d.	1	1	1	1
23	6.63	0	0	0	n.d.	0	0
24	6.81	1	1	1	0	0	0
25	8.1	x	1	1	1	1	1
26	8.4	0	0	1	1	1	1
27	7.1	1	1	1	0	0	0
28	8.8	1	1	1	1	1	1
29	6.1	n.d.	0	0	0	0	0
30	8	0	0	0	1	1	1
31	8	0	0	0	1	1	1
32	8.1	0	0	0	1	1	1
33	8.54	1	1	1	1	1	1
34	8.72	1	1	1	1	1	1
35	7	1	1	1	0	0	0
36	8	0	0	0	1	1	1
37	7.63	1	1	1	0	0	0

Individuals of population 07-407 were subsequently tested with two sets of markers linked to either *Rpi-dlc1* or *Rpi-dlc2* with each set containing three markers (Table 2).

Within that population 14 plants contained markers linked to *Rpi-dlc1* and *Rpi-dlc2*. Eight plants contained only *Rpi-dlc1*, and eight only *Rpi-dlc2*. Seven plants did not contain any marker linked to the resistance gene. Comparison of molecular and phenotypic data showed that the individuals carrying *Rpi-dlc1* and *Rpi-dlc2* had without any exception the highest resistance level observed. Plants containing only *Rpi-dlc2* were classified to either very resistant group or resistant group. Plants with only *Rpi-dlc1* were classified to resistant or slightly susceptible group. Plants that did not contain any of the markers linked to resistance genes were either classified as slightly susceptible or susceptible to late blight, with exception of one plant that was classified as resistant (Figure 2).

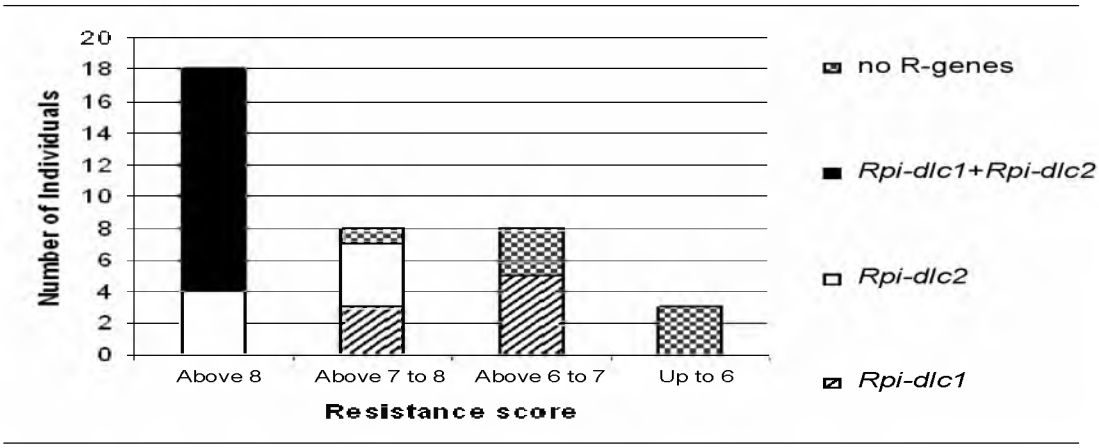

Figure 2. Distribution of resistance scores of individuals in relation to lack or presence of one or two resistance genes. On the X axis the resistance scores ranging from 1-9 where 1 means susceptibility to late blight and 9 immunity to late blight. On the Y axis the number of individuals assigned to each phenotypic class containing one two or none of resistance genes.

Discussion

The goal of this study was to identify the genetic factors responsible for the resistance against *P. infestans* observed in wild *S. dulcamara* accessions (Golas et al. submitted; 2009c). In this paper we have described identification and field evaluation of the second gene that confers resistance against late blight, named *Rpi-dlc2*. Additionally a population containing *Rpi-dlc1* and *Rpi-dlc2* was tested under field condition and showed increased resistance in individuals containing both genes.

Results obtained during the mapping of *Rpi-dlc1* indicated a possibility that additional *Rpi* genes might be present in *S. dulcamara* (Golas et al. submitted; 2009b).

This study shows that at least two resistance genes are present in bittersweet and efficiently protect this plant against late blight attacks during the growing season. Additionally, identification of *Rpi-dlc2* strengthens the idea that *S. dulcamara* might solely rely its defense on *R*-genes rather than other defense strategies that are different from what has been documented for potato or tomato (Thipyapong et al. 1995). DLA and field experiments showed that *Rpi-dlc2* stops *P. infestans* at an early stage of infection and the pathogen is not able to close the life cycle. Furthermore, resistance assays revealed a black/white segregation, similar to reactions of other *R*-genes identified in *Solanum*. By contrast, *Rpi-dlc1* seems not to behave in the same way (Golas et al. submitted; 2009b). The finding of *Rpi-dlc2* opens a new source of *R*-genes that might significantly vary from genes of American origin previously identified. Such different class of resistance might be of great value for breeders and ultimately may be used in breeding programs. Alternatively further investigation of bittersweet *R*-genes may provide us with an insight on the strategies this wild *Solanum* employs to defend it self against *P. infestans*. This knowledge as well might be exploited to protect cultivated potato. However, further studies are needed to understand why *R*-genes apparently are still very successful in *S. dulcamara* whereas it was not during the history of potato breeding. Another question is also if *Rpi-dlc1* and *Rpi-dlc2* are part of yet much larger array of *R*-genes employed by *S. dulcamara*, like in the case of e.g. *S. demissum* or *S. bulbocastanum* where several *R*-genes were identified (Gebhardt and Valkonen 2001).

It is very likely that *Rpi-dlc1* and *Rpi-dlc2* are recognizing effectors of *P. infestans*. The presence of such genes in bittersweet possibly plays a role in shaping the virulence spectrum of *P. infestans* populations. Although natural late blight infections of *S. dulcamara* are seldom observed (Cooke et al. 2002; Flier et al. 2003a) plants susceptible to some extent are attacked. In that way, rare combinations of virulence genes might be maintained in *P. infestans* populations. Yet, screen with a set of effectors similar to those described in Vleeshouvers et al. (2008) that enabled discovery and functional profiling of late blight *R*-genes and *Avr*-genes, could also unravel the broadness of recognition of virulence factors detected by both genes from *S. dulcamara*. Alternatively we might discover whether similar effectors are recognized by European *Solanum* species and its American relatives.

Genetic mapping of *Rpi-dlc2* is still in progress, due to several problems. The main one was the low level of homology between the *S. dulcamara* chromosome region containing *Rpi-dlc2* and the corresponding region in potato and tomato. Although AFLP proved to be a powerful tool enabling fast identification of markers linked to the resistance loci, the sequences obtained from these markers, however, were difficult to work with.

Informations from blast analyses, so useful in mapping *Rpi-dlc1*, were misleading indicating false positive chromosome positions of the sequenced markers. Apparent differences on DNA level between potato/tomato and *S. dulcamara* increased mistakes even further. Secondly, converted AFLP markers into PCR based markers did not amplify useful fragment in potato mapping population or tomato insertion lines that could be used in determining the chromosomal position. Moreover, the amount of markers that could be used for mapping was limited due to the lack of *S. dulcamara* sequence informations. Furthermore, potato/tomato markers were highly inefficient as only about thirty percent could amplify a corresponding region of *S. dulcamara*. Finally, a low level of polymorphism between parents from which crosses 05-150 and 07-410 were derived made mapping process slow and laborious. Primers designed on potato/tomato sequences were not efficient in the amplification of fragments in the high-resolution DNA melting profiling, a powerful tool to detect polymorphisms. Similarly to *S. dulcamara*, another non tuber bearing species, *S. caripense* Dunal, was also investigated for late blight resistance and was also found to be highly homozygous. This required the development of SNP-based allele specific markers (Nakitandwe et al. 2007a), which aided the construction of a map that has positioned the *Rpi* gene on chromosome 9 (Nakitandwe et al. 2007b). A similar approach might help mapping *S. dulcamara Rpi-dlc2* gene as well. Likewise, a set of molecular genetic tools such as saturated genetic map, mapping population, EST library or physical map of *S. dulcamara* should be considered a valuable development for future studies on *S. duclamara Rpi* genes.

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6

Summary and concluding remarks

The research described in this thesis comprises of an insight into the genetic structure of *Solanum dulcamara*, its role in late blight epidemiology and unravels some of the genetic factors responsible for the resistance to *Phytophthora infestans* (Mont.) de Bary.

In the beginning, research was mainly focused on wild relatives of potato belonging to *Solanum* section *Solanum* species like *S. americanum* Mill., *S. scabrum* Mill., *S. villosum* Lam., and *S. nigrum* L., with *S. dulcamara* L. belonging to different section being treated marginally. *Solanum* section *Solanum* species like *S. nigrum* can grow as weeds in potato fields, exhibiting a high level of resistance to *P. infestans* and therefore were considered as a non-host for the potato late blight pathogen and valuable source of resistance (Colon et al. 1993; Kamoun et al. 1999). However to start genetic studies susceptible parent is required thus, main concern and huge effort was put in identification of susceptible genotypes that could be used in the future to develop segregating populations suitable for mapping studies. However, despite of testing both in field and in DLA numerous accessions of section *Solanum* species we had failed to detect a suitable susceptible parent. Some susceptible phenotypes could be observed in mentioned species, though they were not reproducible over the experiments. Still, most promising ones were self pollinated or crossed with other identified within that group susceptible plants. Obtained populations up to the third generation were tested in resistance assays using several *P. infestans* isolates, without any success of finding traces of increased susceptibility caused by increased dosage of recessive resistance alleles. We concluded that despite occasional reports of infections (Hirst and Steadman 1960; Flier et al. 2003) susceptibility in that plant group either does not exist or is very unique, but following other authors this plant species should be considered as an alternative hosts for the *P. infestans*. Most of the *Solanum* section *Solanum* species have a high genetic complexity due to high ploidy level, thus attempts of trying to establish susceptibility through mutant approach would require generation of large populations screened for stable susceptible mutants. Also virus induced gene silencing even though it is more efficient technique used in loss-of-function studies could fail due to high accumulation of resistant genes that would mask the effect of silencing. Recently susceptible genotypes were identified for *S. nigrum* in detached leaf assay (Lebecka 2008) and currently are being used to investigate resistance present in these nightshades. Initial phenotypic segregation in developed populations of *S. nigrum* suggests that resistance against late blight might also be conferred by a single dominant resistance gene(s) (Lebecka 2009).

Lack of appropriate plant material within the section *Solanum* and identification of a clear susceptible bittersweet decided that this species and its sources of resistance will be investigated.

Solanum dulcamara was a species poorly understood; therefore one of the first aims was to gain more insight into the genetic structure present between accessions and individuals.

Although bittersweet collection was already present in the seed bank of Radboud University Botanical and Experimental Garden with about 35 entries representing 10 countries, to have a more complete cross section, new accessions were intensively collected prior the final experiment described in the chapter 2. This resulted in broadening of the collection to about 165 accessions that represent primarily European accessions, with few representatives from North America and New Zealand. A part of this collection was used to measure genetic variation (chapter 2) and study interactions between bittersweet and *P. infestans* presented in chapter 3. Some selected individuals were used as parents in crosses that led to identification of two resistance genes described in chapter 4 and chapter 5. For some of the accessions, additional informations like precise collection site and habitat were plants or seeds were collected were also made available. This added to our understanding of distribution of *S. dulcamara* in natural environment and its coexistence with *P. infestans* in nature.

To gain insight into the genetic variation present in European accessions of *S. dulcamara* described in chapter 2 about half of the available accessions were tested. The main purpose of investigation the genetic variation was to identify the eventual “isles of diversity” that could be present in populations of *S. dulcamara*. They could have arisen due to sheer physical distance or natural barriers, separating patches of populations. Such separated populations could have slightly different genetic composition worthwhile of investigating. Greater diversity would certainly influence the selection of individuals for experiments carried out later on. However lack of differences on the genomic level to that extent was a surprise considering that this plant is morphologically quite diverse. Also, wide distribution and plasticity of this species had very little reflection on the DNA level. To study bittersweet accessions we have applied AFLP as it is very efficient technique to generate random polymorphic markers covering entire genome. Such approach ensured us that variation (or lack of it) did not come from a single gene or small region of the genome (Meud and Clarke 2007).

High similarity between accessions and individuals of *S. dulcamara* was encountered during mapping of the *Rpi-dlc2*. Both resistant and susceptible parents were genetically \alike because of similar geographical origin of accessions from which individuals were selected for crosses. This uniformity between parents was the major problem during the mapping as this probably caused notorious lack of polymorphism. Although lack of polymorphism is not unique for *S. dulcamara* as similar problems were encountered during the construction of the genetic map of *Solanum caripense* (Nakitandwe et al. VI International Solanaceae Conference) we did not have other choice of the susceptible genotypes than those already used in construction of F_1 and F_2-BC_1.

Additionaly none of the sequence obtained from AFLP fragments shared significant similarity to those of potato or tomato what made mapping of *Rpi-dlc1* easy.

Finaly none of AFLP fragments linked to *Rpi-dlc2* could be amplified either in introgression lines of tomato or potato mapping populations used by us.

Even though lack of choice among susceptible genotypes created enormous problems during mapping, still identification of these individuals was the most important discovery that influenced the entire thesis. These two individuals coded 947500001-2 and 947500001-5 were used in every chapter either to study genetic variation (chapter 2) or as a reference of disease severity (chapter 3). Ultimately they were used as pollen donors to the resistant parents in crosses allowing developing of segregating populations that enabled us to identify two resistance genes (chapter 4, chapter 5). To our knowledge these are the first susceptible to that extent genotypes of bittersweet. Although susceptibility in *S. dulcamara* was already reported (de Bary 1876; Cooke et al. 2002; Flier et al. 2003) none of the authors described totally collapsed plants as a result of *P. infestans* infection. Also during this study despite testing of great amount of accessions we never came across of an individual even close to the phenotype exhibited by 947500001-2 and 947500001-5. These two susceptible individuals probably do not contain any of the resistance genes that are present in the populations of *S. duclamara*. Most of the bittersweet accessions displayed either immunity to late blight or high level of resistance where infections are relatively limited and occurred mostly on young stems and older leaves. Although we also identified susceptible individuals all of them in contrast to 947500001-2 and 947500001-5 were able to recover later in the season when the disease pressure lowered. These semi-susceptible plants probably contained a weak gene like for example described by us *Rpi-dlc1* or other yet unknown. Alternatively their resistance could depend on the weather conditions and overall fitness of the plant and the pathogen or maybe its virulence pattern. Presence of susceptibility among *S. dulcamara* is rare and similarly to bittersweet, exceptions from resistance are probably present in every species of *Solanum* which are now considered as non-host, thus consistent screening of individual will result in identification of susceptible genotype like in case of *S. nigrum* (Lebecka 2009).

Improving the genetic resistance to late blight is currently a major issue in breeding new varieties of potato. Since major gene resistance from *S. demissum* is considered nondurable, it can be argued that either durable resistance should be from different and distant sources or disease reducing resistance of agronomical level should be investigated. In current breeding programs several wild *Solanum* species have been found to carry valuable resistance to late blight and *S. dulcamara* was never regarded as possible and maybe a valuable source of resistance to potato late blight. In this study we provide the first data about resistance in this species and its performance under experimental field conditions. Both genes described in this thesis are very dissimilar to each other in terms of speed and efficiency with which they protect bittersweet.

The first one, *Rpi-dlc1* acts as a weak gene in contrast to *Rpi-dlc2*, exhibiting a clear resistance. In case of *Rpi-dlc1* despite increased resistance of individuals that contain this gene plants are not immune and attacks occurs mainly on young stems and leaves damaging the plants. This is not acceptable in breeding and this gene alone has little value for commercial use. This weak response can be caused by not complete affinity of R-gene towards the *P. infestans* caused maybe by other functions that this gene originally has in the cell. In contrast, *Rpi-dlc2* provides high field resistance level and plants carrying it were free of disease during the entire period of experimentation. This shows clear R-gene that evolved to protect agains *P. infestans*. Additionally field experiment on population where some individuals were containing one, two or none of the resistance genes showed that further increase of resistance level can be obtained by stacking them in to the single plant. Thus ongoing effort should be made to identify more genes from that species and asses them under field conditions. Moreover isolation and sequencing of identified genes will enable us to compare them to already known genes. It is very intriguing how different or similar they are to those one identified in American species. Additionally isolated genes can be transformed into the *S. tuberosum* background and tested for their effectiveness. Polyculture of resistance genes with different level of resistance and originating from different donor species is a very promising approach and possibly offers free of disease cultivars in future. Off course a big question is how these cultivars will perform when cultivated on commercial scale. If this approach will turn to failure like early excitement with *S. demissum* than breeding for non host resistance or influencing the pathways can be still an option.

Both pathogen and its host are constantly interacting with each and two theories for evolution of resistance are currently proposed: the trench ware fare and arms race. Both theories require interaction between pathogen and the host thus scientific explanation for presence of resistance gene against alien (for Europe) pathogen is also intriguing. In chapter 4 we already discussed some possible explanations for presence of R-genes in *S. dulcamara* that maybe have partially answered that question. But more we know about *S. dulcamara* and its interaction with biotic stresses will allowed us to find a true answer.

During the project tests of accessions revealed a remarkably high variation in response to P. infestans inoculation. This as we know now is partially attributed to the presence of resistance genes. At least two of them work and efficiently protect S. dulcamara against P. infestans attacks. As we showed combination of two genes adds to immunity of the plant as genotype containing two genes did not show any variation to the resistance but just immunity. This might indicate that no more genes are working there Old world species of *Solanum* though resistant to late blight were never studied in details or considered as source of resistance to late blight. As our results indicated, European *Solanum* species are using resistance genes to defend themselfs against late blight.

It is still possible that there are more genes than two described in this thesis as wide response spectrum was obserwed across accessions as described in chapter 3. Althoug isolation and sequencing of the *Rpi-dlc1* and *Rpi-dlc2* is an obvious but not easy goal for the future, whe sould try to investigate additional genotypes.

Solanum dulcamara proved to be an easy plant to work with due to its many advantages. Production of large amounts of seeds of subsequent generations is very easy. Fast and imidiate germination of seeds makes the time needed for next generations very short. This combined with limited selfing, easy to work flowers; occuring in large numbers on the plants generates a possibility to work with bulk amounts. Additionaly it is frost resistant perenial, growing on poor soil thus valuable enotypes can be easily maintained for a long period of time on small space. It performed relatively well in labolatory assays and field experiments were each year populations or accessions were screened for resistance to *P. infestans*. Easy genetics and available protocols for transformation (Curtis et al. 2000) make this plant a promising for genetic manipulations.

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7

Summary

Solanum dulcamara (Solanaceae) commonly named bittersweet is a diploid European species recently dispersed to other parts of the world. It belongs to section *Dulcamara* of subgenus *Potatoe*, which size and content is poorly defined. This wild relative of cultivated potato was recognized early as a host for *Phytophthora infestans*, the cause of late blight in potato and tomato and later for other potato pathogens and pests. Throughout in North-West Europe, *S. dulcamara* apparently may interfere with potato disease epidemiology, but knowledge on this aspect and on the species in general is scarce.

The first aim of this thesis (**chapter 2**) was to measure genetic variation and correlation between genomic similarity and geographical provenance among a collection of accessions of *S. dulcamara* that broadly represented this European species. To prevent risk of under-sampling and to cover the width of the variation a set of 79 accessions (245 individuals) representing about 35 collection sites was used. Genomic profiling using three AFLP primer combinations yielded a dataset containing 288 polymorphic markers used to generate Simple Matching dendrograms.

Analyses of *S. dulcamara* populations revealed an average similarity above 80%. This high similarity was in agreement with results of an experiment that indicated that cross pollination rather than selfing predominates in nature on this species. Likely, this leads to increased gene flow between populations. The general characteristics of this plant; being a perennial, widely occurring in natural and inhabited habitats and having efficient vegetative reproduction as well as seed dispersal by animals could additionally contribute to gene exchange among populations. Although the genetic variation in this species is low, the similarity between accessions collected outside The Netherlands was lower than the similarity among the Dutch accessions, reflecting to some extent the geographical separation. Also the clustering of accessions from different regions of Europe and partial clustering of accessions from The Netherlands indicated some structure on the genetic level. Still, it was concluded that European *S. dulcamara* is a solid species that shows a rather limited tendency to sub-cluster according to geographical derivation or ecological niches. Morphological differences e.g. growth habit, leaf size or shape do not correlate clearly to groups of similar genotypes identified in the AFLP dendrogram. The high genetic similarity does not interfere with the species' capacity to adapt to various contrasting habitats such as dry dune areas and wet lands. *Solanum dulcamara*, as observed in the vicinity of cultivated potato, is a recorded host for *P. infestans*.

In **chapter 3** an attempt is described to evaluate the frequency of natural late blight infections by monitoring four sites where *S. dulcamara* was abundantly present throughout 2006 and 2007. Plants were growing either along canals close to fields where potatoes were grown organically or in dune area where potato cultivation is exceptional. Despite frequent evaluations of plants for symptoms and collection of blemished leaves, the presence of the pathogen could not be confirmed. This lack of positive result indicated that susceptibility in this species is rather unique. Alternatively, very susceptible plants are eliminated at an early stage of development. Either way, the impact of *S. dulcamara* on overall late blight epidemiology was considered to be minimal. To further study the resistance of *S. dulcamara* to *P. infestans*, sets of 21 and 52 accessions was screened under field conditions in 2006 and 2007, respectively.

Results indicated that the sampling region might have some influence, as more susceptible genotypes were observed among individuals collected in dune areas. By contrast, the most resistant plant was collected in a cultivated potato area. Moreover, the pattern of natural infections that occurred on accessions growing in the open at the Radboud University Botanical and Experimental Garden was similar to what has been described for some *Solanum* species in Mexico and it was concluded that it is unlikely that *S. dulcamara* is responsible for initiating late blight epidemics in The Netherlands. This species seems also not to serve as a reservoir for overwintering inoculum from the previous cropping season. Yet, it became clear that bittersweet is a good host for *P. infestans* and that it can suffer from attacks of the pathogen similarly as susceptible potato cultivars do.

In **chapter 4,** a characterization of the genetic factors responsible for the elevated level of resistance in some *S. dulcamara* accessions against *P. infestans* is described. Initial identification of a susceptible individual suitable for genetic studies among 14 *S. dulcamara* accessions allowed the development of three F_2-BC_1 populations that segregated for resistance to late blight in a detached leaf assay and under field conditions. Genetic profiling of one of these populations using 128 AFLP primer combinations enabled identification of three markers linked to the resistant phenotype; two at 5cM, and one at 2cM distance from a gene designated as *Rpi-dlc1*. Blast analysis of sequenced markers allowed confirming the position of the gene on distal end of the long arm of chromosome 9. One of the populations was subsequently enlarged to 302 individuals and enabled the construction of more detailed genetic map of the *Rpi-dlc1* locus that was compared to an equivalent region in the genome of *S. tuberosum*.

The other two segregating populations were also found to contain the *Rpi-dlc1* locus. Tests for the broadness of resistance responses using a set of seven *P. infestans* isolates indicated that additional *Rpi* genes in this species could be present.
Some possible explanations for the presence of *R*-genes in *S. dulcamara* were proposed. They could be recruited from a general reservoir of functional *R* genes that must have been present at the time of arrival of *P. infestans* around 1845, and during subsequent seasons with late blight epidemics, or *R*-genes like *Rpi-dlc1* could operate against different, yet unknown pathogens, but with a pleiotropic effect on late blight. Finally, but less likely, it is possible that *P. infestans* was already present in Europe long before 1845. Hence, *S. dulcamara* and other European solanaceous plant species could already have been evolutionarily adapted.

In **chapter 5**, it is described that additional screening of 20 accessions of *S. dulcamara* revealed one resistant individual in accession A54750069. A F_1 population, derived form a cross between this plant and a susceptible genotype, containing 63 individuals was found to segregate in a 1:1 fashion. The presence of *Rpi-dlc1* as a cause of the observed resistance was excluded by marker analysis. Subsequent AFLP profiling using 196 primer combinations enabled identification of 5 markers linked to this second resistance gene putatively named *Rpi-dlc2*. Additionally, a population segregating for the *Rpi-dlc1* and *Rpi-dlc2* resistance genes was created that indicated that individuals containing both genes are more resistant to *P. infestans* under field conditions than those with either one gene or none. This study shows that at least two resistance genes are present in bittersweet, which efficiently can protect this plant against late blight attacks during the growing season. Additionally, identification of *Rpi-dlc2* strengthens the idea that *S. dulcamara* might solely rely its defence on *R* genes. The finding of *Rpi-dlc1* and *Rpi-dlc2* opens a new source of *R* genes that might significantly vary from *R*-genes of American origin previously identified in potato and tomato. However, efforts of positioning the *Rpi-dlc2* gene on the genetic map using a potato mapping population, tomato introgression lines, SSR markers, CAPS markers, or SNP detection failed. Likely, several aspects negatively affected the mapping efficiency, e.g. a low level of homology between the *S. dulcamara* chromosome region containing *Rpi-dlc2* and the corresponding regions in potato and tomato, lack of sequence specific amplification of co-segregating *S. dulcamara* markers in the reference potato mapping population and tomato insertion lines, as well as the low level of heterozygosity and polymorphism between the parents from which populations segregating for *Rpi-dlc2* were derived. Therefore, further research into this interesting European solanaceous species would greatly benefit from establishment of a general genomic knowledge consisting of, for example, a classical genetic reference map and genomic sequences for EST's, COS-markers, BAC-ends etc.

Solanum dulcamara (Solanaceae) is een diploïde Europese plantensoort die in het Nederlands Bitterzoet wordt genoemd en die vrij recentelijk naar andere werelddelen is verspreid. De soort behoort tot de sectie *Dulcamara* van het subgenus *Potatoe*, waarvan de omvang en samenstelling slechts in beperkte mate is vastgesteld. Deze wilde verwant van de cultuuraardappel was al lang bekend als gastheer voor *Phytophthora infestans*, welke in aardappelen en tomaat de aardappelziekte veroorzaakt en in een later stadium ook voor andere ziekten en plagen van aardappelen. Het lijkt erop dat *S. dulcamara* overal in Noordwest Europa een rol zou kunnen spelen in de epidemiologie van aardappelziekten hoewel de kennis van dit aspect van deze plantensoort in het algemeen beperkt is.

De eerste doelstelling van dit onderzoek (**hoofdstuk 2**) was om de genetische variatie binnen een collectie van herkomsten van *S. dulcamara* te meten die een brede afspiegeling van deze Europese soort was en om de correlaties te berekenen tussen waarden voor genomische verwantschap (similariteit) en geografische herkomst. Om het risico van ondervertegenwoordiging te voorkomen en ook de gehele breedte van de variatie te beslaan werd een groep van 79 herkomsten (245 individuen) gebruikt die circa 35 verzamelplaatsen representeerden. Genomische profilering met een drietal AFLP primer combinaties leverden een dataset op die 288 polymorfe merkers bevatte waarmee Simple Matching boomdiagrammen werden gegenereerd. De analyse van *S. dulcamara* populaties leverde een gemiddelde waarde voor similariteit op van boven de 80 procent. Deze hoge waarde was in overeenstemming met de resultaten van een proef die een aanwijzing opleverde dat bij deze soort in de natuur vooral kruisbevruchting en niet zozeer zelfbevruchting plaatsvindt. Dit leidt waarschijnlijk tot verhoogde genetische uitwisseling tussen populaties. De algemene eigenschappen van deze plant, zijnde overjarig, wijdverspreid voorkomend in zowel natuurlijke en bebouwde habitat, met een efficiënte vegetatieve vermeerdering alsook de verspreiding van de zaden via dieren, zouden kunnen bijdragen tot genetische uitwisseling tussen populaties. Ofschoon de genetische variatie in deze soort gering is, werd gevonden dat de similariteit tussen herkomsten die buiten Nederland zijn verzameld kleiner is dan de similariteit van Nederlandse herkomsten, hetgeen tot op zeker hoogte een geografische scheiding weergaf. Ook de groepering van herkomsten uit verschillden delen van Europa enerzijds en gedeeltelijke groepering van herkomsten uit Nederland duidde op enige structuur op genetisch niveau. Evengoed werd geconcludeerd dat *S. dulcamara* uit Europa een duidelijke plantensoort is die een geringe tendens vertoont om te sub-groeperen naar geografische afstamming of ecologisch niche.
Morfologische verschillen zoals groeiwijze, bladgrootte of bladvorm vertonen geen duidelijke correlatie met de groepen zoals die in het AFLP-boomdiagram waren geïdentificeerd.

Het hoge niveau van genetische similariteit beïnvloedt kennelijk niet het vermogen van de soort om zich aan verscheidene contrasterende milieus zoals droge duinen en waterrijke gebieden aan te passen. *Solanum dulcamara*, zoals geobserveerd in de buurt van aardappelpercelen, is bekend als gastheer van *P. infestans*.

In **hoofdstuk 3** wordt een poging beschreven om gedurende 2006 en 2007 op vier plekken waar *S. dulcamara* volop voorkwam, het optreden van natuurlijke infecties met de aardappelziekte vast te stellen. De planten groeiden langs kanalen in de buurt van velden waar op biologische wijze geteelde aardappelen groeiden of juist in duingebieden waar aardappelteelt uitzonderlijk is. Ondanks veelvuldige evaluatie van de planten voor symptomen en het verzamelen van bladeren met vlekjes kon de aanwezigheid van de ziekteverwekker niet vastgesteld worden. Dit gebrek aan positief resultaat wees erop dat vatbaarheid in deze soort vrij uniek is. Een andere verklaring is dat erg vatbare planten vroeg in hun ontwikkelingstadium geëlimineerd worden. Hoe dan ook, het belang van *S. dulcamara* in de algehele epidemiologie van de aardappelziekte werd beschouwd minimaal te zijn. Om de resistentie van *S. dulcamara* tegen *P. infestans* nader te bestuderen werden in 2006 en 2007 respectievelijk 21 en 52 herkomsten getoetst onder veldomstandigheden.

De resultaten wezen erop dat de bemonsteringsregio enige invloed zou kunnen hebben, aangezien meer vatbare genotypes werden geobserveerd tussen de individuen verzameld in duingebieden. Daarentegen werd de meest resistente plant verzameld in een gebied met aardappelteelt. Bovendien was het patroon van de natuurlijke infecties in accessies groeiend buiten in de vollegrond, in de botanische tuin van de Radboud Universiteit, vrijwel gelijk aan hetgeen beschreven is voor enkele *Solanum* soorten in Mexico. Hieruit werd geconcludeerd dat het onwaarschijnlijk is dat *S. dulcamara* verantwoordelijk is voor de aardappelziekte epidemieën in Nederland. Deze soort lijkt ook niet te fungeren als reservoir waarin inoculum van het afgelopen teeltseizoen overwintert. Evengoed werd duidelijk dat Bitterzoet een goede gastheer is voor *P. infestans* en dat de plant in gelijke mate als vatbare aardappelrassen te lijden kan hebben van aantasting door deze pathogeen.

In **hoofdstuk 4** wordt een karakterisering beschreven van de genetische factoren die verantwoordelijk zijn voor het verhoogde niveau van resistentie tegen *P. infestans* in enkele *S. dulcamara* accessies. Nadat in eerste instantie een vatbaar individu kon worden geïdentificeerd binnen 14 *S. dulcamara* accessies dat geschikt was voor genetische studies, konden drie F_2-BC_1 populaties worden gemaakt die splitsten voor resistentie tegen de aardappelziekte zowel in een blaadjestoets als ook onder veldcondities.

De genetische profilering van één van deze populaties met 128 AFLP primercombinaties maakte de identificatie mogelijk van drie merkers die gekoppeld waren met het resistente fenotype; twee op 5cM en één op 2cM afstand van een gen dat *Rpi-dlc1* werd genoemd. Met behulp van blastanalyse van gesequenste merkers werd de positie van het gen aan het distale einde van de lange arm van chromosoom 9 bevestigd. Eén van de populaties werd daarop vergroot tot 302 individuen en dit maakte het mogelijk om een meer gedetailleerde kaart van het *Rpi-dlc1* locus te construeren die vergeleken werd met een overeenkomstig stuk in het genoom van *S. tuberosum*. Ook in de andere twee splitsende populaties werd de *Rpi-dlc1* locus gevonden. Toetsen voor de breedte van de resistentie, gebruikmakend van een set van zeven *P. infestans* isolaties, gaven aanwijzingen dat additionele *Rpi*-genen in deze soort aanwezig zouden kunnen zijn.
Enkele mogelijke verklaringen voor de aanwezigheid van *R*-genen in *S. dulcamara* werden gegeven. Zij zouden mogelijk gerekruteerd kunnen worden uit een algemeen reservoir van functionele *R*-genen welke aanwezig moet zijn geweest ten tijde van de binnenkomst van *P. infestans* rond 1845 als ook gedurende de opeenvolgende seizoenen met epidemieën van de aardappelziekte. Of *R*-genen, zoals *Rpi-dlc1,* zouden tegen verschillende nog onbekende pathogenen blijken te kunnen werken en daarbij een pleiotropisch effect hebben tegen de aardappelziekte. Tenslotte, hoewel onwaarschijnlijk, is het mogelijk dat *P. infestans* reeds aanwezig was in Europa lang voor 1845. Daardoor zouden *S. dulcamara* en andere Europese soorten uit de Solanaceae reeds evolutionair aangepast kunnen zijn geweest.

In **hoofdstuk 5** wordt beschreven hoe additionele screening van 20 accessies van *S. dulcamara* één resistent individu opleverde afkomstig van accessie A54750069. Een F_1 populatie, ontstaan uit een kruising tussen deze plant en een vatbaar genotype en die 63 individuen bevatte, splitste ongeveer uit in een 1:1 verhouding. De aanwezigheid *van Rpi-dlc1* als oorzaak van waargenomen resistentie werd uitgesloten met merker analyse. Daaropvolgende AFLP profilering met gebruikmaking van 196 primer combinaties maakten de identificatie mogelijk van 5 merkers die gekoppeld waren aan dit tweede resistentie gen dat voorlopig *Rpi-dlc2* genoemd werd. Daarna werd een populatie gecreëerd die segregeerde voor de *Rpi-dlc1 en Rpi-dlc2* resistentiegenen die aanwijzingen verschafte dat individuen met beide genen meer resistent waren tegen *P. infestans* onder veldcondities dan die met één of geen enkel gen. Dit onderzoek toonde aan dat tenminste twee resistentiegenen in Bitterzoet aanwezig zijn die de plant gedurende het groeiseizoen effectief kunnen beschermen tegen de aardappelziekte .De identificatie van *Rpi-dlc2* versterkt bovendien het idee dat *S. dulcamara* zijn verdediging wel eens uitsluitend kon baseren op *R*-genen.

De vondst van *Rpi-dlc1* en *Rpi-dlc2* vormt een nieuwe bron van *R*-genen die beduidend zouden kunnen afwijken van *R*-genen van Amerikaanse oorsprong zoals eerder geïdentificeerd in aardappel en tomaat. Echter, pogingen om het *Rpi-dlc2* gen te positioneren op de genetische kaart, waarbij gebruik gemaakt werd van een aardappelkarteringspopulatie, introgressielijnen van tomaat, SSR merkers, CAPS merkers, of SNP-detectie, faalden. Waarschijnlijk werd de effectiviteit van karteren negatief beïnvloed door verscheidene factoren, zoals een laag niveau van homologie tussen het chromosoomgebied *S. dulcamara* dat *Rpi-dlc2* bevat en de overeenkomstige gebieden in aardappel en tomaat, gebrek aan sequentiespecifieke amplificatie van co-segregerende *S. dulcamara* merkers in de referentie aardappelkarteringspopulatie en insertielijnen van tomaat, alsmede het lage niveau van heterozygotie en polymorfie tussen de ouders waarvan de populaties die splitsten voor *Rpi-dlc2* waren afgeleid. Verder onderzoek naar deze interessante Europese Solanaceae soort zou veel baat hebben bij het tot stand brengen van een algemene genomische kennisbasis bestaande uit bijvoorbeeld een klassieke genetische referentiekaart en genomische sequenties voor EST's, COS-merkers, BAC-uiteinden, enzovoorts.

Curriculum vitae

Acknowledgements

Tomasz Michal Golas was born on the 30th of March 1978 in Warsaw, Poland.
From 1985 to 1993 he followed his Primary Education at Primary School nr. 325 in Warsaw. Between 1993 and 1998 he pursued Secondary Level Education at Agricultural Technical School in Warsaw.
Between 1998 and 2003 he followed a Higher Education at Warsaw Agricultural University. He obtained his master degree in June 2004. In August 2003 until April 2004 he went as research student to Wageningen University, Department of Nematology.
In September 2004 he started his PhD at the Radboud University in Nijmegen, Institute of Water and Wetland Research, Department of Plant Cell Biology. The outcome of his study is presented in this thesis. From September 2008 he continued his work as a postdoctoral researcher at the same department.

At this point I would like to thank the people that helped me during the time spent working on my PhD and without whom this thesis would not exist.

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Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Tomasz Michal Golas
Date: 28 January 2010
Group: Plant Cell Biology, Radboud University Nijmegen

1) Start-up phase	*date*
► **First presentation of your project**	
Identification of new sources of resistance in wild accessions of	Nov 12, 2004
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Safe handling with radioactive materials and sources	Nov 19, 2007
Subtotal Start-up Phase	*3.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD Student Days**	
EPS PhD student day, Nijmegen	Jun 02, 2005
EPS PhD student day, Wageningen	Sep 19, 2006
EPS PhD student day, Wageningen	Sep 13, 2007
► **EPS Theme Symposia**	
Theme 4 Symposium "Genome Plasticity, Wageninger	Dec 09, 2005
Theme 4 Symposium "Genome Plasticity, Nijmeger	Dec 08, 2006
Theme 4 Symposium "Genome Plasticity, Leider	Dec 07, 2007
► **NWO Lunteren days and other National Platforms**	
NWO-ALW Lunteren meeting	Apr 04-05, 2005
NWO-ALW Lunteren meeting	Apr 03-04, 2006
NWO-ALW Lunteren meeting	Apr 02-03, 2007
NWO-ALW Lunteren meeting	Apr 07-08, 2008
► **Seminars (series), workshops and symposia**	
Seminar Sophien Camoun	Sep 15, 2005
IWWR symposium day	Nov 25, 2005
IWWR symposium day	Nov 30, 2006
IWWR symposium day	Nov 29, 2007
IWWR symposium day	Nov 27, 2008
► **Seminar plus**	
► **International symposia and congresses**	
First Solanaceae Genome Workshop, Wageningen, The Netherlands	Sep 19-21, 2004
Solanaceae Conference Madison, USA	Jul 23-27, 2006
The 5th Solanaceae Genome Workshop, Koln, Germany	Oct 12-16, 2008
► **Presentations**	
CBSG summit	Nov 11, 2004
CBSG summit	Feb 21-22, 2005
CBSG summit	Sep 08, 2005
CBSG summit	Mar 07, 2006
CBSG summit	Oct 05, 2006
CBSG summit	Feb 07, 2007
CBSG summit	Aug 31, 2007
IWWR meeting	Nov 29, 2007
CBSG summit	Mar 13-14, 2008
Solanaceae Conference Madison, USA (poster)	Jul 23-27, 2006
► **IAB interview**	Sep 13, 2007
► **Excursions**	
Subtotal Scientific Exposure	*17.1 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Molecular phylogenies: reconstruction and interpretatior	Oct 18-22, 2004
Bioinformatics - A User's Approach	Mar 13-16, 2007
Summer School Signaling in Plant Development and Defence: Towards	Jun 19-21, 2006
Summer School On the Evolution of Plant Pathogen Interactions: from F	Jun 18-20, 2008
► **Journal club**	
participated in discussion group 'Plant Cell Biology	Sep 2004-Sep 2008
► **Individual research training**	
Agrico Research B.V., practical lab training (15 days)	Feb 2008
Subtotal In-Depth Studies	*10.5 credits**

4) Personal development	*date*
► **Skill training courses**	
Career Perspectives	Mar 26, Apr 02,09,16,23, May 07, 2008
Dutch, level 4	Sep 27-Dec 11, 2007
► **Organisation of PhD students day, course or conference**	
Co-organizer of PhD student day 2006	Sep 2006
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*6.3 credits**

TOTAL NUMBER OF CREDIT POINTS*	**36,9**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by th Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*